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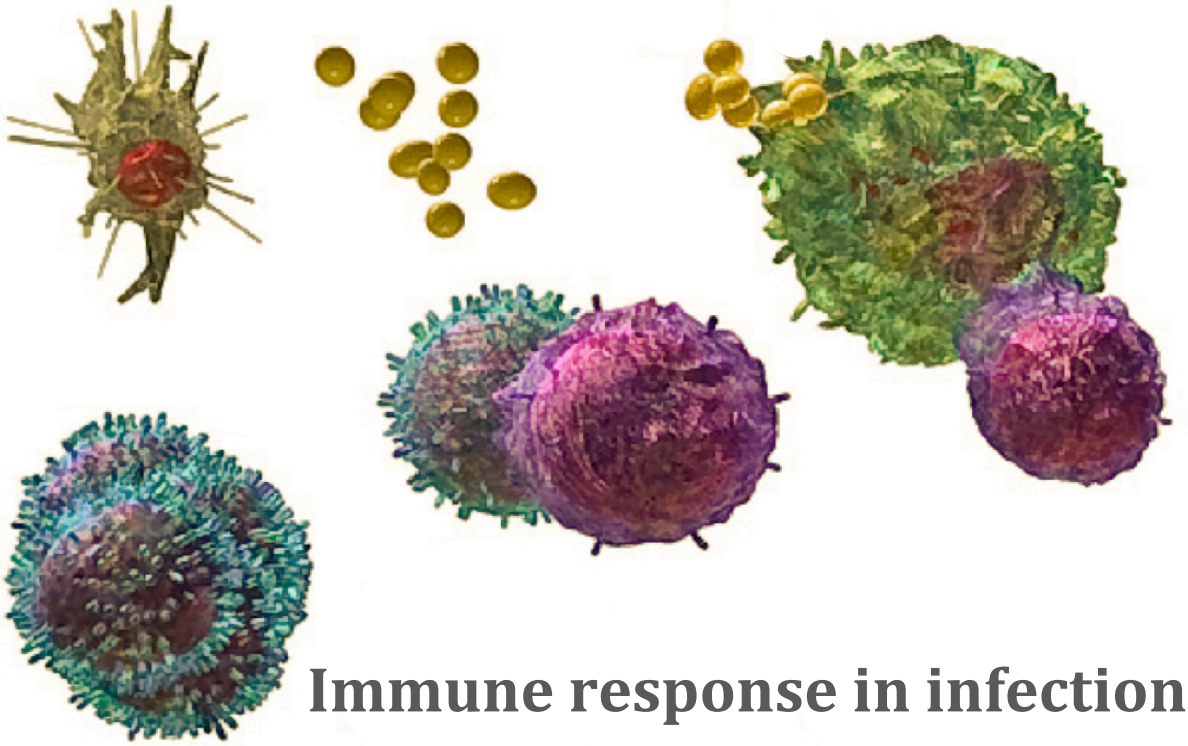
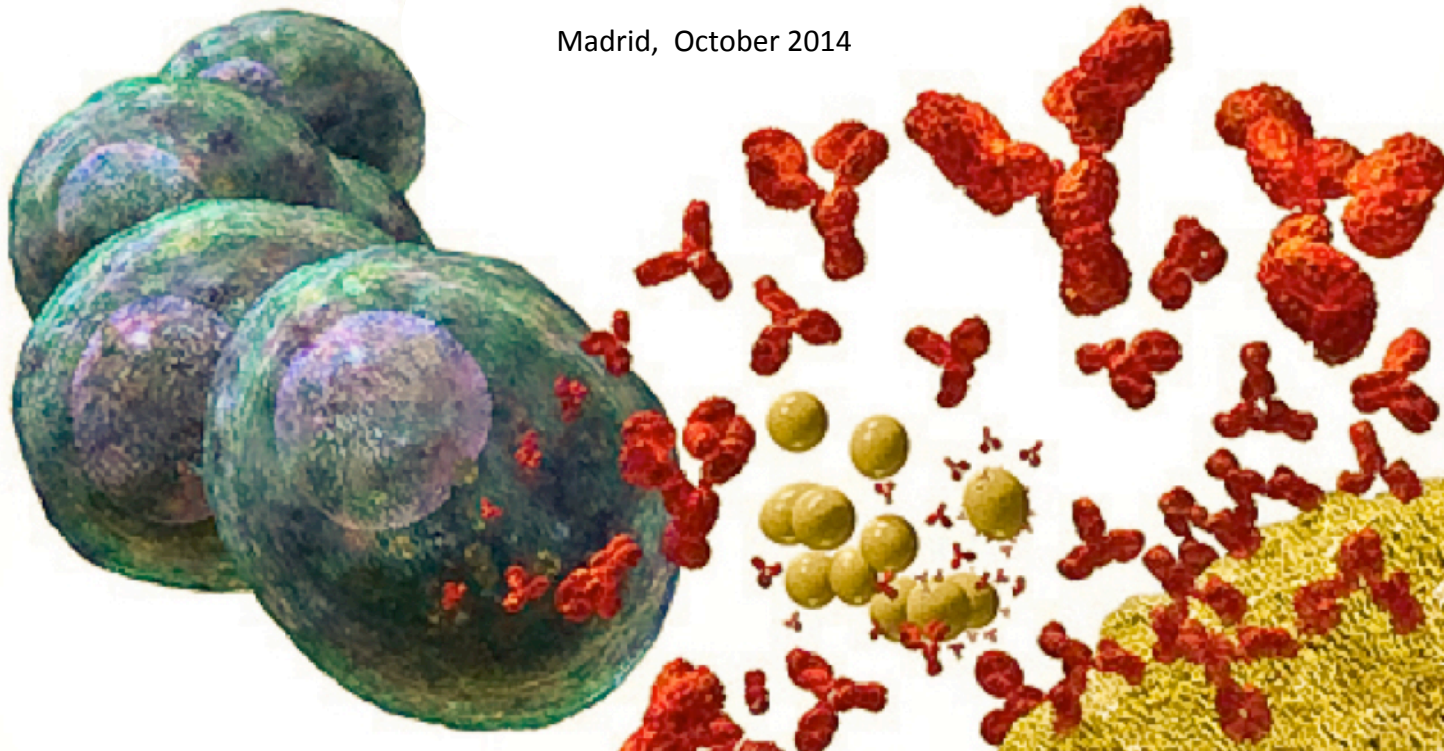


Immune response in infection: Role of human NKG2H receptor and study of HLA-E restricted lymphocytes

Daniela Dukovska

PhD thesis dissertation

Madrid, October 2014



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The research presented in this thesis was carried out in the Department of Immunology and Oncology of the National Centre for Biotechnology / CSIC under the direction of **Mar Vales Gomez Ph.D. and Hugh T. Reyburn PhD.**

¨La página escrita nunca recuerda todo lo que se ha intentado, solo lo poco que se ha conseguido¨

Antonio Machado

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Abstract
Presentación

The NKG2 family is one of the multiple families of immune receptors encoded in the human genome that contains members with activating and inhibitory potential. The CD94/NKG2A inhibiting and CD94/ NKG2C activating receptors are mainly expressed on NK cells and subsets of $CD8^+$ T cells and have been shown to play an important role in regulating immune responses against infected and tumour cells. However the biology of other members of the NKG2 family has been much less explored. Strikingly, both CD94/NKG2A and CD94/NKG2C can bind the same ligand; the non-classical HLA class I molecule HLA-E loaded with nonamer peptides derived from the signal sequence of classical MHC class I molecules or with peptides derived from other proteins, including pathogen proteins, to form peptide/HLA-E/β2-microglobulin trimers that can be recognized by specific T cells for immune activation.

The experiments described in this thesis have addressed two aspects of the biology of the CD94/NKG2 – HLA-E system:

In the first part of this work, the NKG2H receptor, an as yet poorly characterized member of the NKG2 family has been studied in detail. NKG2H was expressed on low numbers of peripheral blood lymphocytes, mainly on $CD3^+CD56^+$ and $CD3^+CD8^+$ T cells. Activation of NKG2H expressing cells markedly reduced the activation and proliferation of the other T cells in the culture via induction of apoptosis. This suppressive activity was characteristic of NKG2H but not for NKG2A or NKG2C expressing cells and did not depend on the release of soluble factors or recognition of MHC class I molecules. A marked increase in the proportion of T and NK cells expressing $NKG2H^+$ but not $NKG2A^+$ or $NKG2C^+$ cells was observed after co-culture of PBMCs, from healthy HCMV seronegative individuals, with HCMV infected fibroblasts. The expansion of $NKG2H^+$ cells depended on contact between PBMCs and HCMV infected fibroblasts and separation of these cells led to the loss of NKG2H expression.

In the second part of the thesis, a possible role of *Mycobacterium tuberculosis* (Mtb) – peptide specific, HLA-E restricted cells in the immune response of bladder cancer patients receiving BCG immunotherapy was studied. Low frequencies of $CD8^+$ T cells reactive with the Mtb-peptide/HLA-E tetramers were observed in the peripheral blood of these patients.

Our data are the first to describe the populations of cells that express the CD94/NKG2H receptor, the effects on lymphocyte function elicited by receptor ligation and a possible role for CD94/NKG2H in immune responses to viral infection. Further, the HLA-E tetramer staining method has been shown to be applicable for the detection and characterization of Mtb specific HLA-E restricted T cells during BCG therapy in bladder cancer patients, although this approach could be improved by use of a larger library of Mtb-peptide/HLA-E complexes.

NKG2 es una familia de receptores inmunes que engloba algunos miembros con potencial activador y otros inhibidores, como sucede con otras muchas otras familias de receptores inmunes codificadas en el genoma humano. Los heterodímeros CD94/NKG2A y CD94/NKG2C, inhibidor y activador respectivamente, se han estudiado en profundidad en los últimos años. Estos receptores se expresan sobre todo en células Natural Killer (NK) y subpoblaciones de linfocitos T CD8+ y se ha demostrado que desempeñan un importante papel en la regulación de la respuesta inmune generada frente a células infectadas y tumorales. Sin embargo, la biología de algunos miembros de la familia NKG2 no se ha estudiado todavía. Tanto CD94/NKG2A como CD94/NKG2C reconocen el mismo ligando: la molécula de MHC de clase I no clásica HLA-E, cargada con péptidos nonaméricos derivados de la secuencia señal de otras moléculas de histocompatibilidad clásicas; aunque también se ha descrito que HLA-E puede presentar péptidos derivados de otras proteínas, incluyendo proteínas de patógenos, formando trímeros péptido/HLA-E/β2- microglobulina que pueden ser reconocidos por células T específicas.

Los experimentos descritos en esta tesis estudian dos aspectos de la biología del sistema CD94/NKG2 – HLA-E:

En la primera parte de este trabajo, se estudia en detalle el todavía poco conocido receptor NKG2H. Se muestra que este receptor se expresa en la superficie celular de un porcentaje muy bajo de células efectoras, principalmente $CD3^+CD56^+$ y T $CD3^+CD8^+$. La activación de linfocitos a través de NKG2H redujo de manera considerable la activación y la proliferación de otras células T en el cultivo, mediante inducción de apoptosis. Este efecto supresor era característico de NKG2H pero no de células que expresaban NKG2A o NKG2C, y no dependía de la liberación de factores solubles o del reconocimiento de MHC de clase I. Tras el cultivo de células de sangre periférica, proveniente de donantes sanos seronegativos para HCMV con fibroblastos infectados con HCMV, se observó un incremento notable en la proporción de células NK que expresaban NKG2H pero no NKG2A o NKG2C. La expansión de células positivas para NKG2H dependía del contacto entre las células de sangre periférica y los fibroblastos infectados, y la separación de ambas células daba lugar a la pérdida de expresión de NKG2H.

En la segunda parte de esta tesis, se estudia el posible papel de péptidos específicos de Mycobacterium tuberculosis, restringidos por HLA-E, en la respuesta inmune generada por pacientes de cáncer de vejiga recibiendo inmunoterapia basada en BCG. Utilizando tetrámeros HLA-E/ péptido Mtb se encontraron frecuencias bajas de células positivas en sangre periférica de los pacientes.

Nuestros datos son los primeros en describir el fenotipo, las características funcionales y el posible papel en las respuestas inmunes frente a infecciones virales desempeñadas por el receptor CD94/NKG2H. Más aún, se demuestra que el uso de tetrámeros de HLA-E constituye un método aplicable para teñir células de sangre periférica y evaluar la presencia de células T restringidas por péptidos específicos de Mtb en el caso de los pacientes y cáncer de vejiga, aunque este procedimiento se puede mejorar utilizando un repertorio más amplio de complejos péptido Mtb / HLA-E.

Abbreviations

7-AAD	7-aminoactinomycin d
APC	Allophycocyanin
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
Ct	Cycle threshold
CTLA4	Cytotoxic T-lymphocyte antigen 4
Cy	Cyanine
DMEM	Dulbecco modified eagle medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
FACS	Fluorescent-activated cell sorter
FBS	Fetal bovine serum
Fc	Antibody crystallizable region
Fig.	Figure
FITC	Fluorescein isothiocyanate
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-E	HLA class I histocompatibillity antigen, alpha chain E
HS	Human serum
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer-cell immunoglobulin receptor
Lamp1	Lysosomal-associated membrane protein
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
Min	Minutes
mRNA	Messenger RNA
NK	Natural killer cell
NKT	Natural killer T cell
p	P-value
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Polyhydroxyalkanoate
PKC	Protein kinase C
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium

RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TCR	T cell receptor
TNF	Tumour necrosis factor

Introduction

The Inhibition / activation paradigm in immunology

1. Introduction

NK cells were, for a long time, thought to be regulated by the 'missing-self' hypothesis (Ljunggren and Karre, 1990). This theory proposed that NK cells were constitutively active, but inhibited via inhibiting receptors and their interaction with MHC class I. In the case of down-regulation of MHC class I such as in virally infected cells and cancerous cells, inhibition would be lost, leading to activation of the NK cell's function, namely, killing infected or defective cells within the body. However, eventually it was discovered that NK cells also express activating receptors, and that, additionally, inhibiting receptors were not restricted to the NK cell subset, but were expressed on many other immune cell subsets such as B cells, monocyte/macrophages, dendritic cells (DCs) and T cells. Further, it was discovered that there were specialized families of receptor pairs/families with both activating and inhibiting members (Fig. 1). This has led to a new paradigm of homeostasis where any given cell is controlled by a balance of both inhibiting and activating signals. When the balance is tipped to one side or the other, the cell is moved into action, with inhibition or activation being dependent on which way the balance swings.

1.1 ¨Paired receptors¨

Cell surface receptors are integral membrane proteins that interact with either soluble factors or ligands on other target cells and regulate cell function. The term ¨Paired receptors¨ refers to closely related membrane proteins often expressed on immune cells. They are generally encoded by different genes, but have very similar extracellular ligand-binding regions and have both activating and inhibitory members (Akkaya et al. 2013). CD28 and CTLA-4 are probably the best-studied example of paired co-activating/co-inhibitory receptors. These molecules are expressed on T cells and bind the same ligands (i.e., CD80 and CD86) to deliver opposing positive and negative signals (Lenschow et al. 1996; Krummel et al. 1996[(1)(2)]; Chambers et al. 1999). For NK cells, the inhibitory receptors usually contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) within the cytoplasmic region. Upon ligand binding, immune inhibitory receptors are phosphorylated on the tyrosine residue, recruit phosphatases and inhibit the propagation of activation signals in the lymphocyte on which they are expressed. In contrast, the activating receptors have a short cytoplasmic region lacking ITIM elements, but containing a charged amino acid residue (usually Arg or Lys) in the transmembrane region, that mediates association with transmembrane adaptor proteins bearing either an immunoreceptor tyrosine-based activation motif (ITAM) (CD247, FcεRγ, DAP12), or a PI3 kinase/Grb2 binding motif (DAP10). ITAM motifs, upon ligand binding, lead to the association of kinases such as Syk and Zap 70 and transmitting of activating signals into the cell (Lanier et al. 2000; Love et al. 2000). The extracellular regions of NK receptors usually contain either immunoglobulin superfamily domains or C-type lectin-like domains. For example, the KIR and CD94/NKG2 that recognize

MHC class I ligands, contain both activating and inhibitory receptors within the families (Lanier et al. 1998). Paired activating and inhibitory receptors families have also been identified on other cell types (Ali et al. 2014). So, the immune system can be considered to be tightly regulated by the balance between the activating and inhibitory signals initiated by these paired receptors, and deregulation of this balance is often associated with autoimmunity, allergy, and various infectious diseases.

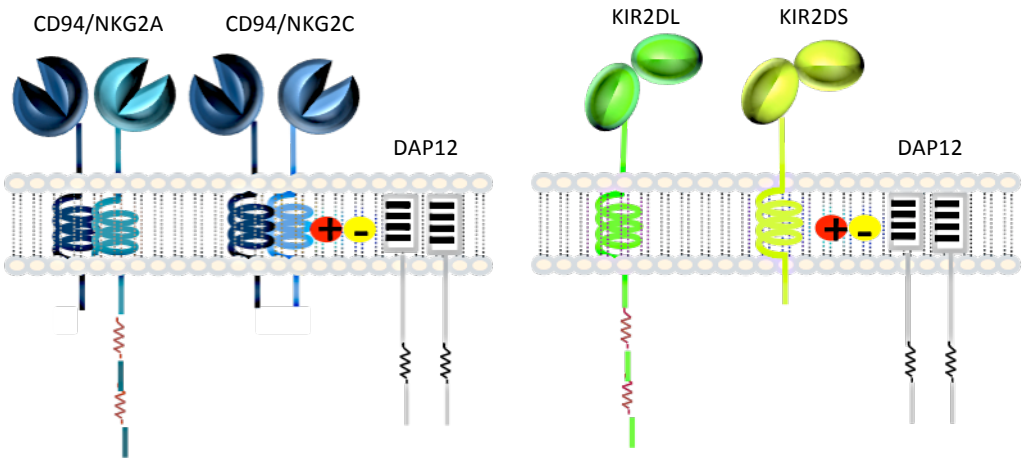

Fig. 1: Paired receptors. Paired receptors consist of activating and inhibitory receptors having highly homologous extracellular ligand binding regions. The inhibitory receptors possess an immunoreceptor tyrosine-based inhibitory motif (ITIM) within the cytoplasmic region. In contrast, the activating receptors have short cytoplasmic regions and a positively charged residue (Arg or Lys) in the transmembrane domain, which allows association with an adaptor protein possessing an immunoreceptor tyrosine-based activation motif (ITAM). The left panel displays the C-type lectin-like receptors including the inhibitory heterodimer CD94/NKG2A and activating heterodimer CD94/NKG2C. The right panel illustrates the two-Ig-like domain KIR proteins including KIR2DS activating and KIR2DL inhibitory receptor. The ITIM motif is shown in red whereas the ITAM motif is shown in black.

1.2 Integration of signals in NK cells

In the 1970s, a population of lymphocytes able to "spontaneously" mediate cytotoxicity, without prior exposure to the target, was described in the spleen of unmanipulated mice and rats and in the peripheral blood of humans (Rosenberg et al. 1974; Herberman et al. 1975[(1)(2)]). These initial observations were rapidly confirmed and the term natural killer (NK) cell was added to the immunological lexicon (Kiessling et al. 1975). NK cells are a population of lymphocytes (approximately 10% of peripheral blood mononuclear cells, PBMCs), distinct from B and T cells and located at the crossroads between innate and adaptive immunity. They play important roles in the coordination of both tumour immunosurveillance and the immune response against pathogens. Diverse functional activities have been associated with NK cells, including ¨natural¨ cytotoxicity and antibody-dependent cell-mediated cytotoxicity both dependent on perforin/granzyme release, as well as the capacity of NK cells to produce a range of cytokines including tumour-necrosis factor (TNF-α) and interferon-γ (IFNγ) and chemokines that help to

generate and sustain an inflammatory environment (Trinchieri et al. 1989). NK cells thus participate in an early defence system via direct elimination of virally infected or transformed cells through target-cell lysis, via activation of dendritic cells (DCs) and via modulation of the response of adaptive lymphocytes (T cells and B cells) through the release of soluble factors. Human NK cells are phenotypically and functionally heterogeneous. Approximately 10% of NK cells found in blood express high levels of surface CD56 and are classified as $CD56^{bright}$. These NK cells do not express CD16 (also known as FcγRIIIA), can produce abundant amounts of cytokines (IFNγ, TNF-α, granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-10 and IL-13) and various chemokines, but have little ability to spontaneously kill tumour cell targets (Nagler et al. 1989; Caligiuri et al. 1990; Cooper et al. 2001). In contrast, the majority of circulating human NK cells show low surface density or ¨dim¨ expression of CD56 ($CD56^{dim}$), do express CD16 at the cell surface, are less efficient cytokine producers (per cell) in response to activation by monokines, but are highly cytotoxic and can spontaneously lyse many tumours and virally infected targets (Lanier et al. 1986; Jacobs et al. 2001; Walzer et al. 2007).

The prevalent view of NK cell activation is that specific NK recognition of infected or transformed cells while maintaining tolerance for ¨self¨, depends on a balance of signals from activating and inhibitory receptors. According to the ¨missing self¨ hypothesis (Karre et al. 1986; Ljunggren et al. 1990), the binding, or not, of NK cell inhibitory receptors to target cell MHC class I (cognate ligands for many NK inhibitory receptors and expressed by virtually every cell in the body) determines NK cell specificity (Karlhofer et al. 1992; Wagtmann et al. 1995; Dohring et al. 1996). The recognition of MHC class I molecules by the NK cell inhibitory receptor leads to the recruitment of phosphatases that block the propagation of signals which lead to target cell killing. Down-regulation of the expression of MHC class I molecules on the target cell surface, with the consequent lack of engagement of the inhibitory receptor and absence of an inhibitory signal favours NK cell activation and killing of the target cell (Moretta et al. 1992[(1)]; Moretta et al. 1997; Ploegh et al. 1998; Algarra et al. 2000). This point of view has been modified recently as it has become clear that a threshold for NK cell responsiveness exists and that the definition of this checkpoint is regulated by a licensing or education process (Shifrin et al. 2014). During this process NK cells not only acquire receptor expression and become self-tolerant, but they are also licensed for effector function. The molecular mechanisms underlying NK cell licensing are poorly understood, but it is generally agreed that interactions between MHC class I molecules and inhibitory receptors are critical for this process (Hoglundet al. 2010).

The recognition that NK cells express a variety of activating receptors led to a modification of the missing-self hypothesis to include the idea that NK cells were not constitutively active, but rather required specific signals for activation that could then be subject to the action of the inhibitory receptors (Yokoyama et al. 1993). It is now widely appreciated that host cells can be modified as a result of cellular stress, viral infection or tumour transformation leading to induction or up regulation at the cell surface of molecules that at physiological conditions are low/not expressed. These molecules can engage activating receptors, such as NKG2D (Bauer et al. 1999), triggering NK cell activation and subsequent elimination of the target cells.

Activating receptors associated with ITAM-containing adaptor proteins (CD3ζ, FcεRIγ and DAP12) transmit strong activation signals into the cell through recruitment of tyrosine kinases Syk and ζ-associated protein (ZAP-70). Such receptors can be further subdivided into two groups. The first group comprises KIRs and CD94/NKG2 family members that are specific for MHC class I molecules, and includes KIR2DS, KIR3DS (binding classical MHC class I), CD94/NKG2C (binding HLA-E) and CD94/NKG2E. These receptors belong to the group of "paired receptors¨ and associate with the ITAM-containing adaptor molecule DAP12. The second group of ITAM-associated receptors includes the activating receptors mainly expressed on NK cells (and responsible for NK-cell non-MHC-restricted natural cytotoxicity): NKp46, NKp30 and NKp44, referred to as natural cytotoxicity receptors (NCR) by their discoverers (Sivori et al. 1997; Vitale et al. 1998; Pessino et al. 1998; Sivori et al. 1999; Pende et al. 1999; Cantoni et al. 1999). NKp44 couples with DAP12, while NKp30 and NKp46 interact with hetero or homodimers of CD3ζ and FcεRIγ. Another receptor in this group is CD16. This receptor mediates antibody-dependent cell-mediated cytotoxicity (ADCC) (Perussia et al. 1983; Takai et al. 1994) and associates with CD3ζ and FcεRIγ.

Another category of activating receptors on NK cells is the ITAM independent receptors, which include NKG2D, CD2, 2B4 and DNAM-1. In humans, the NKG2D receptor associates specifically with the adaptor protein DAP10 (Wu et al. 1999), containing a phosphatidylinositol-3 kinase (PI3K) binding motif. NKG2D expression is not confined only to NK cells, since it is also expressed by virtually all T-cell receptor (TCR) γ/δ^+ and $CD8^+$ TCR α/β^+ cells. The ligands for NKG2D, the MHC class I chain related gene A (MICA), MHC class I chain related gene B (MICB), and UL16-binding protein (ULBP), are expressed on some tumour cells and on infected or stressed cells (Nausch and Cerwenka. 2008). 2B4 (CD244), receptor is expressed by NK cells but also can be found on activated $CD8^+$ T cells, monocytes and granulocytes. Binding of its ligand (CD48), leads to recruitment of SLAM-associated protein (SAP) and Fyn through cytoplasmic immunotyrosine-based switch motifs (ITSMs). (Moretta et al. 1992[(1)]; Nakajima et al. 1999; Tangye et al. 1999; Nakajima et al. 2000; Sivori et al. 2000; Chuang et al. 2001).

As mentioned previously, to avoid killing "self" cells NK cells also express receptors that can trigger inhibiting signals. These receptors can also recognize MHC class I molecules. In humans, three families of such inhibitory NK receptors have been described. One major group is referred to as killer immunoglobulin (Ig)-like receptors (KIR). They possess two or three Ig-domains and each member interacts with a different group of closely related classical HLA class I molecules (HLA-A, HLA-B or HLA-C). For example, HLA-C molecules are the ligands for KIR2D receptors, while subsets of HLA-B and HLA-A molecules are the ligands for KIR3D receptors (Valiante et al. 1993; Litwin et al. 1994; Colonna et al. 1995; Dohring et al. 1996; Pende et al. 1996; Valés-Gómez et al. 1998; Baba et al. 2000; Fan et al. 2001; Ivarsson et al. 2014). A second group of receptors known as LILR (leukocyte immunoglobulin-like receptors) (LIR-1, ILT2) are also members of the Ig superfamily. The third major group of inhibitory receptors expressed on NK cells is the heterodimeric CD94/NKG2 C-type lectin proteins (Houchins et al. 1991; Chang et al. 1995; Lazetic et al. 1996; Brooks et al. 1997; Carretero et al. 1997; Borrego et al. 1998; Braud et al. 1998[(1)]; Lee et al. 1998[(2)]; Bellón et al. 1999; López-Botet et al. 1999; López-Botet et al. 2000[(1)]). The inhibitory receptor from this family is CD94/NKG2A. All of the

inhibitory receptors from each group possess immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails (Bolland et al. 1999; Long et al. 1999; Daëron et al. 2008; Long et al. 2008).

1.3 Integration of signals in T cells

The 1950s, 60s and 70s were an exciting time for immunologists, as major discoveries were made in the field of immunology; the importance of recirculating lymphocytes as mediators of immunity (Gowans et al. 1959; Gowans et al. 1962; Miller et al. 1967), definition of the role of the thymus (Cooper et al. 1967; Cooper et al. 1973) and the existence of two distinct but interacting lymphocyte subsets, one derived from the thymus (T cells) and involved in cellular immunity, and the other derived from the bone marrow (B cells), that produces antibodies (Claman et al. 1966; Miller et al. 1968; Mitchell et al. 1968; Howard et al. 1972; Scott et al. 1972; Bromberg et al. 2004). Immunological compatibility was also defined in the 1970s by the definition of the transplantation antigens – now known as the molecules encoded by the major histocompatibility complex (MHC). The true physiological function of these antigens was unknown up to the point when MHC restriction of T cell recognition was discovered by two young immunologists Rolf Zinkernagel and Peter Doherty, a discovery for which they were awarded the Nobel Prize in Physiology and Medicine in 1996 (Zinkernagel et al. 1973; Oldstone et al. 1973; Zinkernagel et al. 1974; Zinkernagel et al. 1975; Doherty et al. 1975; Zinkernagel et al. 1976; Townsend et al. 1986; Bjorkman et al. 1987). The intense efforts to discover the molecule that mediated specific MHC recognition culminated with the identification of the TCR proteins in late 1982 and early 1983 by the groups of Jim Allison, Ellis Reinherz, John Kappler and Philippa Marrack. A T cell clone-specific disulfide-linked heterodimer of about 45-50 kDa was identified, that comprised an alpha and beta chain and where each chain contained variable and constant regions (Allison et al. 1982; Meuer et al. 1983; Haskins et al. 1983; Acuto et al. 1983; Bigler et al. 1983; Samelson et al. 1983; Hendrick et al. 1984[(1)(2)]; Chien et al. 1984). Soon, a second TCR with structural properties very similar to TCR$\alpha\beta$ was identified and called TCR$\gamma\delta$ (Brenner et al. 1986). The finding that T cells recognize antigen presented by MHC molecules and the development of monoclonal antibody technology, rapidly led to the generation of monoclonal antibodies against the CD3 polypeptides, showing their involvement in antigen recognition (Reinherz et al. 1982). Monoclonal antibodies recognizing the TCR, which inhibited antigen recognition in an antigen-dependent fashion, were first described in 1983, as was the physical and functional relationship between the TCR and the CD3 polypeptides. The mature TCR/CD3 complexes are formed by 6 different polypetides; the TCR$\alpha\beta$ or the TCR$\gamma\delta$ chains and the CD3γ, δ, ε and ζ polypeptides (Marrack et al. 1986; Clevers et al. 1988).

Each T lymphocyte circulating in peripheral blood expresses a unique TCR on the surface as the result of developmental selection upon maturation in the thymus. Two types of T cells appear after this selection according to the TCR expression; the majority express antigen (Ag)-binding $\alpha\beta$ chains in the TCR, which are disulfide-linked heterodimers of Ig superfamily proteins. $\alpha\beta$TCR T cells have a very diverse repertoire of Ag recognition receptors and represent mature T cells that play key roles in driving the adaptive immune response. A small fraction of circulating T cells express $\gamma\delta$ chains, appear to be much less heterogeneous than

αβTCR T cells and play specific roles in determined aspects of the innate immune response to certain pathogens.

Two major surface co-receptor molecules, CD4 and CD8, define two separate T cell lineages with different functions. $CD4^+$ T cells recognize Ag in the context of MHC class II molecules [expressed on so-called antigen presenting cells (APC), such as B cells, macrophages and dendritic cells (DC)] and their key function is to produce cytokines as effector T helper cells. $CD8^+$ lymphocytes are activated by Ag-peptides presented by MHC class I molecules (expressed on all nucleated cells) and act as effector cytotoxic T lymphocytes (CTL).

The functional properties of the T cells allow classification as naive, effector and memory cells, each of which displays considerable diversity in terms of phenotype, function and anatomic/tissue distribution. Naive T cells have not yet encountered foreign Ag and have not been yet activated. When activated, $CD4^+$ T helper cells (Th) can be subdivided into Th1, Th2, Th17, follicular helper T cell (Tfh), induced T-regulatory cells (iTreg) and the regulatory type 1 cells (Tr1) as well as the potentially distinct T-helper 9 (Th9) based on the complex network of specific cytokine signalling and production. (Del-Prete et al. 1991; Trinchieri et al. 2003; Afkarian et al. 2002; Thieu et al. 2008; Kaplan et al. 1996; Glimcher et al. 2000; Veldhoen et al. 2006; Bettelli et al. 2006; Chen et al. 2003; Yoshimura et al. 2011; Vinuesa et al. 2005; Vogelzang et al. 2008; Nurieva et al. 2008). $CD8^+$ T cells also called cytotoxic T lymphocytes (CTL) can be divided into two cell subpopulations termed Tc1 (T cytotoxic 1) and Tc2 (T cytotoxic 1), based on their potential to produce cytokines (Salgame et al. 1991; Maggi et al. 1994; Croft et al; 1994). Tc1 cells secrete IFN-γ and Tc2 produce IL-4, IL2 and TNF-α (Mosmann et al. 1997). Once the antigenic agent is eliminated, most of the effector T cells will undergo apoptosis, although other, few lymphocytes will survive and become long-lived memory T cells (Jacob et al. 1999; Opferman et al. 1999; Dai et al. 2000; Lanzavecchia et al. 2000; Hu et al. 2001; Kaech et al. 2001; Sprent et al. 2001; Bourgeois et al. 2002; Schluns et al. 2002; Sprent et al. 2002; Janssen et al. 2003; Shedlock et al. 2003). Memory T cells, both CD4 and CD8, can be further divided into central and effector memory cells (Sallusto et al. 1999).

The activation of T cells is determined by the presence of distinct signals. The engagement of TCR by antigenic peptides bound to MHC molecules on the surface of APCs provides the so-called ´signal-1´. This signal by itself is not only insufficient for T cell activation but also can lead to apoptosis or a state of antigen-specific non-responsiveness (anergy). For optimal T cell proliferation and acquisition of effector functions a second costimulatory ´signal 2´ is required, provided by a range of surface molecules expressed on APCs. Indeed T cell activation after T cell receptor engagement is influenced by signals from both positive and negative regulatory molecules that can either enhance or inhibit the TCR mediated immune response (Mueller et al. 1989). Four different families, each of which contains costimulatory and coinhibitory molecules, are able to modulate T cell activation: **1.** B7-CD28 family including: CD28 (Jenkins et al. 1991; Boise et al. 1995; Rudd et al. 2003), cytotoxic T-lymphocyte antigen-4 (CTLA-4; CD152) (Brunet et al. 1987; Krummel et al. 1996[(1)(2)]; Walunas et al. 1996), programmed death-1 (PD-1; CD279) (Ishida et al. 1992; Dong et al. 1999; Freeman et al. 2000; Latchman et al. 2001), inducible costimulatory molecule (ICOS; CD278) (Hutloff et al. 1999; Yoshinaga et al. 1999), and B- and T-lymphocyte attenuator (BTLA; CD272) (Watanabe et al. 2003; Chen. 2004; Han et al. 2004; Compaan et al. 2005; Gonzalez et al. 2005). CD28 and

CTLA-4 bind to the same ligands, B7-1 (CD80) and B7-2 (CD86) but the first transmits activating and the second inhibitory signals (Linsley et al. 1994; Collins et al. 2002); **2.** CD2 signalling lymphocyte activation molecule (SLAM) family including: SLAM (CD150), 2B4 (CD244), and CD48 (Boles et al. 2001; Wang et al. 2001); **3.** Ig family including: T cell immunoglobulin mucin-3 (TIM-3) (Rodriguez-Manzanet et al. 2009; Zhu et al. 2011), CD160 (Anumanthan et al. 1998; Agrawal et al. 1999; Cai et al. 2008; Cai et al. 2009), and lymphocyte-activation gene 3 (Lag-3) (Triebel et al. 1990; Huard et al. 1994; Workman et al. 2004; Grosso et al. 2007); and **4.** TNF-receptor superfamily including: CD27 (Denoeud et al. 2011). Studies have shown that $CD8^+$ T lymphocyte activation can also be modulated by receptors that are perhaps more often associated with NK cell activity (Vilches et al. 2002; Raulet et al. 2004). These receptors include members of the KIR family (Huard et al. 2000), NKG2D (Bauer et al. 1999; Groh et al. 2001) and the CD94/NKG2 receptors (Mingari et al. 1998[(1)(2)]; Uhrberg et al. 2001; Young et al. 2001; Jabri et al. 2002; Vivier et al. 2004, Mónica et al. 2005).

As a complex biological element, each step of the signalling pathway initiated by TCR engagement is subjected to both positive and negative regulation. For example, one of the first biochemical consequences of TCR binding is activation of Lck (Veillette et al. 1988) and Fyn, an Src-family protein tyrosine kinases (PTK) (Hermiston et al. 2002). Lck itself is further regulated positively and negatively by CD45 protein tyrosine phosphatase and COOH-terminal Src kinase (CSK) respectively (Thomas et al. 1995; Latour et al. 2001). Once Lck is activated, ITAMs of the CD3 chain are phosphorylated creating docking sites for the tandem Src homology 2 (SH2) domains of the Syk PTK ZAP-70 (Reth 1989; Chan et al. 1991; Chan et al. 1992). After recruitment to the TCR, Zap-70 is also phosphorylated and activated by SRC-family kinases. Active Zap-70 then phosphorylates several downstream substrates, initiating a cascade of signalling pathways that result in nuclear transcriptional changes. Molecules, called adaptor proteins are another important players in these signalling cascades (Peterson et al. 1998; Rudd. 1999; Samelson. 1999). These adaptors lack enzymatic activity, but possess multiple binding sites and modules that bind to other proteins and can function as either positive or negative regulators of T cell signalling. Examples of adaptors with a positive effect include: growth factor receptor-bound protein-2 (GRB-2), the linker for activation of T cell (LAT) (Finco et al. 1998; Zhang et al. 1998; Weber et al. 1998), GRB-2-related adaptor downstream of Shc (GADS) (Yoder et al. 2001) and the SH2-domain-containing leukocyte protein of 76 kDa (SLP-76) (Jackman et al. 1995; Motto et al. 1996). Adaptors with negative regulatory functions include: phosphoproteins associated with (glycosphingolipid-enriched micro domains) GEMs (PAG) (Brdickova et al. 2001; Davidson et al. 2003), the SH-2-interacting transmembrane adaptor protein (SIT) (Hubener et al. 2001) and the downstream of tyrosine kinases (DOK). A number of others adaptors such as the adhesion and degranulation promoting adaptor protein (ADAP) (Geng et al. 2001), the Src kinase-associated phosphoprotein of 55 kDa (SKAP-55) (Marie-Cardine et al. 1997) and the Wiskott-Aldrich syndrome protein (WASP) have regulatory effect on the cytoskeleton, on adhesion and on the ability of T cells to form conjugates with antigen-presenting cells (APCs).

2. The CD94/NKG2 C-Type lectin family of receptors.

CD94 (20-30 kDa) is a type II membrane glycoprotein that forms disulphide-linked heterodimers with different members of the NKG2 family. CD94/NKG2 receptor complexes are expressed predominantly on NK cells and a subset of $CD8^+$ T cells and heterodimers containing NKG2A and NKG2C recognize and bind the non-classical MHC class I molecule HLA-E (HLA class I histocompatibillity antigen, alpha chain E). Orthologous of the CD94/NKG2 family of NK cell receptors have been identified in humans, chimpanzees, orangutans, rheus monkeys, and rodents (Lanier et al. 1998; Long et al. 1997; Guethlein et al. 2002; Houchins et al. 1991; Lohwasser et al. 1999; Berg et al. 1998; La Bonte et al. 2000; La Bonte et al. 2001; Khakoo et al. 2000). These receptors, together with KIR, include some members that can either initiate or inhibit cellular activation therefore by functional criteria they are divided into activating and inhibitory receptors. CD94/NKG2 receptors have been proposed to be important in NK cell tolerance to self, to play an important role in NK cell development, and to contribute to NK cell-mediated immunity to certain infections including human cytomegalovirus.

2.1 CD94/NKG2 gene cluster

Human NK cells express a number of receptors that belong to the C type lectin-like superfamily, such as NKR-P1 (Lanier et al. 1994), members of the NKG2 family (Houchins et al. 1991; Yabe et al. 1993), CD69 (Lopez-Cabrera et al. 1993), and CD94 (Chang et al. 1995). The genes coding for these molecules form part of the NK cell complex (NKC) and are clustered together, in humans, on the short arm of chromosome 12p13 (Fig. 2). The distal to proximal position of these loci are: NKR-P1, CD69, CD94, NKG2D, NKG2F, NKG2E/H, NKG2C and NKG2A/B. The physical distance spanned by the NK gene complex in humans ranges between 0.7 and 2.4 megabases and is flanked by genetic markers D12S397 and D1S89 (Renedo et al. 1997; Suto et al. 1997; Glienke et al. 1998; Sobanov et al. 1999;).

In 1990, Houchins and coworkers identified a number of genes that were expressed preferentially in NK cells, especially those encoding membrane receptors (Houchins et al. 1990). In this report, they identified 12 groups of cross-hybridizing cDNA clones and one of these, NK group 2 (NKG2), was shown to be expressed only in NK cells of the panel of cells that they examined. One year later, they reported that NKG2 was actually a group of closely related transcripts that encode a series of type II membrane proteins, designated NKG2-A, -B, -C, -E and –D (Houchins et al. 1991). Comparison of the peptides encoded by each member of these group, showed that NKG2-A and NKG2-C had high homology in the extracellular part but less throughout the intracellular and transmembrane segments, NKG2-B peptide was a splice variant of NKG2-A and NKG2-E that encodes a 261 amino acid long protein, was 95% similar to NKG2C over the first 191 amino acid residues (Adamkiewich et al. 1993). Finally NKG2-D was the most distantly related of all, having only 20% sequence homology with the other members of the group. Structurally, NKG2 proteins showed sequence similarity to Ca^{2+}-dependent (C-type)

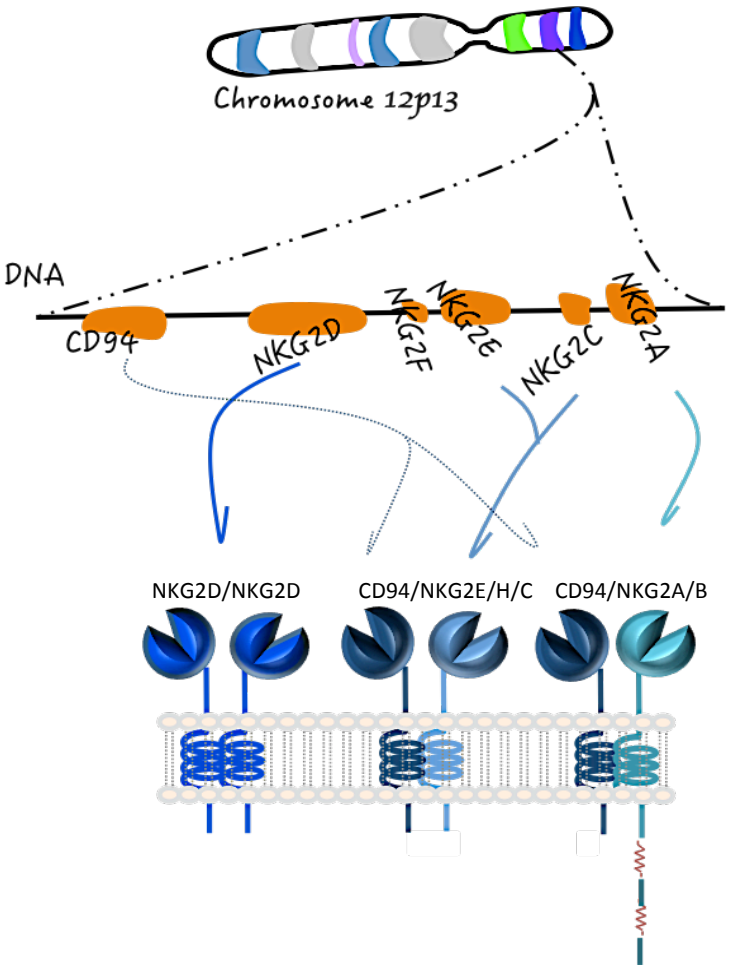

Fig. 2: Chromosomal location of the CD94/NKG2 gene cluster in humans. The CD94/NKG2 family gene cluster is located on the short arm of chromosome 12p13. Five NKG2 genes give rise to seven proteins, two of which NKG2D and NKG2F do not form a heterodimer with CD94 molecule. NKG2D is structurally the most different from the other members of this family and is expressed at the cell surface as homodimer. NKG2B is a splice variant of NKG2A and both function as inhibitory receptors making heterodimers with CD94 molecule. NKG2H is an alternatively spliced variant of the NKG2E transcript and together with NKG2C protein all three possess activating potential. The putative NKG2F protein has a short extracellular region, which does not contain a C-type lectin domain, but it is similar to NKG2C in that a lysine residue is found in the transmembrane region.

lectin domain proteins possessing a type II membrane orientation. The basic structure of these proteins is that of an intracellular N-terminal domain, a transmembrane domain and an extracellular carbohydrate-recognition domain, which is the ligand binding part of the molecule (Adamkiewicz et al. 1994). NKG2A and its splice variant NKG2B have been shown to possess two ITIMs in their cytoplasmic domains. The NKG2C molecule lacked ITIMs, but instead had a positively charged residue within the transmembrane region that allowed association with the ITAM-containing adaptor molecule DAP12 (Lanier et al. 1998). In 1997, a new member of the NKG2 family of receptors was identified by Plougastel et al. The NKG2F gene localizes 25 kb from NKG2A, does not contain any lectin domain but has a charged residue in the transmembrane region allowing interaction with DAP12 (Kim et al. 2004) and an ITIM like motif in

the cytoplasmic tail. Shortly afterwards, Bellón et al. 1999 published results describing a TCR$\alpha\beta^+$, CD8$^+$ T cell clone (TCC; K14B06) that expressed an activating CD94 heterodimer that could trigger redirected lysis in a TCR-independent manner. Biochemical and molecular characterization of the heterodimer identified a novel NKG2 molecule, named NKG2H. Comparison of the nucleotide sequence of the NKG2H cDNA with NKG2 genomic sequences revealed that NKG2H is an alternative splice form of NKG2E in which intron VI has not been removed and that does not contain exon VII (Fig. 3). Like NKG2C, NKG2H has a short cytoplasmic tail, lacks ITIMs but does possess a charged lysine residue in the transmembrane domain that could permit interaction with the DAP12 adaptor molecule.

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NKG2E           MSKQRGTFSEVSLAQDPKWQQRKPKGNKSSISGTEQEIFQVELNLQNASLNHQGIDKIYDCQGLLPPPEKLTAE 115
NKG2H           MSKQRGTFSEVSLAQDPKWQQRKPKGNKSSISGTEQEIFQVELNLQNASLNHQGIDKIYDCQGLLPPPEKLTAE 60
NKG2C           MNKQRGTFSEVSLAQDPKRQQRKPKGNKSSISGTEQEIFQVELNLQNPSLNHQGIDKIYDCQGLLPPPEKLTAE 60
NKG2A           MDNQGVIYSDLNLPPNPKRQQRKPKGNKSSILATEQEITYAELNLQKASQDFQGNDKTYHCKDLPSAPEKLIVG 60
CD94            ------------------------------------------------------------MAVFKTTLWRLISG
                                                                               : ..  :*

NKG2E           VLGIICIVLMATV—LKTIVLIPFL              EQ--NNSSPNARTQKARHCG
NKG2H           VLGIICIVLMATV—LKTIVLIPFL              EQ--NNSSPNARTQKARHCG
NKG2C           VLGIICIVLMATV—LKTIVLIPFL              EQ--NNFSPNTRTQKARHCG
NKG2A           ILGIICLILMASV—VTIVVIPSTLI             -QRHNNSSLNTRTQKARHCG
CD94            TLGIICLSLMATLGILL             KNSFTKLSIEPAFTPGPNIELQKDSDCC
                  *****: ***::  :                      :   . . * . **  .*

NKG2E           HCPEEWITYSNSCYYIGKERRTWEESLQACASKNSSSLLCIDNEEEMKFLASILPSSWIGVFRNSSHHPWVTINGLAFKHEIKDSDH-- 177
NKG2H           HCPEEWITYSNSCYYIGKERRTWEESLQACASKNSSSLLCIDNEEEMKFLASILPSSWIGVFRNSSHHPWVTINGLAFKHEIKDSDH-- 177
NKG2C           HCPEEWITYSNSCYYIGKERRTWEESLLACTSKNSS-LLSIDNEEEMKFLASILPSSWIGVFRNSSHHPWVTINGLAFKHKIKDSDN-- 176
NKG2A           HCPEEWITYSNSCYYIGKERRTWEESLLACTSKNSS-LLSIDNEEEMKFLSIISPSSWIGVFRNSSHHPWVTMNGLAFKHEIKDSDN-- 178
CD94            SCQEKWVGYRCNCYFISSEQKTWNESRHLCASQKSS-LLQLQNTDELDFMSSSQQFYWIGLSYSEEHTAWLWENGSALSQYLFPSFETF 120
                 * *:*: *  .**:*..*::**:**   *:*::** ** ::* :*:.*::      ***:  ...* .*:  ** *:.: :  * .

NKG2E           AERNCAMLHVRGLISDQ-CGSSRIIRRGFIMLTRLVLNS----------------- 240
NKG2H           AERNCAMLHVRGLISDQ-CGSSRIIVSISFRIKALELAVHQIKFYICSNRNDIMIA 257
NKG2C           AELNCAVLQVNRLKSAQ-CGSSMIYHCKHKL------------------------ 231
NKG2A           AELNCAVLQVNRLKSAQ-CGSSIIYHCKHKL------------------------ 233
CD94            NTKNCIAYNPNGNALDESCEDKNRYICKQQLI---------------------- 179
                   **   : .     : *..         :
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Fig. 3: Alignment of NKG2 family members and CD94. (*) indicate identical residues; (:) indicate highly similar residues; (.) indicate partially similar residues. The alignment was done using ClustalW2-Multiple Sequence Alignment program.

Earlier, in 1990, immunological studies, carried out in parallel to the cloning studies outlined above, led to the description of a novel cell surface disulfide-linked dimer initially called Kp43, that was preferentially expressed by NK cells, TCR$\gamma\delta^+$ T lymphocytes and some CD8$^+$CD56$^+$TCR$\alpha\beta^+$ T cell clones (Aramburu et al. 1990; Aramburu et al. 1991). Functionally, monoclonal antibodies specific for Kp43 could modulate NK cell-mediated cytotoxicity and cytokine production (Aramburu et al. 1993). Molecular cloning of the cDNA encoding Kp43 (now termed CD94) (Chang et al. 1995), led to the identification of a single-copy gene, located in chromosome 12p13.1, encoding a type II membrane glycoprotein with a C-type lectin-like domain and a short cytoplasmic tail. All the cDNA clones isolated displayed an identical ORF (open reading frame), encoding for a 180-amino acid polypeptide with a short intra-cytoplasmic domain (7 amino acids), which contains a charged residue (Lys) in the transmembrane region. However, the biochemical studies in polyclonal NK cells indicated that Kp43 was assembled as a

disulfide-linked dimer, comprising two subunits (Lopez-Botet et al. 1995). In addition, immunoprecipitation analysis of NK cells obtained from different donors showed two forms of CD94 molecule corresponding to molecular size of 43kDa (p43) and 39kDa (p39), providing further evidence for the structural heterogeneity of these proteins (Perez-Villar et al. 1995; Perez-Villar et al. 1996). These observations were consistent with functional data showing that the specific anti-Kp43 (CD94) mAb might either inhibit or activate NK cell function, but were difficult to reconcile with the invariant nature of the cloned CD94 transcript.

A solution to this dilemma was provided by Phillips et al. 1996, who reported that a polyclonal antiserum raised against the cloned CD94 protein did not react in Western blotting with Kp43, strongly suggesting that CD94 and Kp43 were different molecules; Kp43 (CD94) was renamed ¨CD94-associated protein¨. A plausible candidate for 43kDa glycoprotein (p43) was the recently identified C-type lectin protein NKG2A, the only molecule of this family that contained cytoplasmic ITIMs and had the predicted molecular weight. Subsequent experiments confirmed that the p43 subunit was encoded by NKG2A that assembled with CD94 to constitute the functional receptor complex (Plougastel et al. 1996; Lazetic et al. 1996; Carretero et al. 1997).

NKG2B, the splice variant of NKG2A, as well as other members of the NKG2 multigene family including NKG2C (corresponding to the previously detected p39 subunit) and NKG2E/H also require assembly with CD94 to generate functional heterodimeric receptors. NKG2D, as mentioned previously, is distantly related to the other members of the NKG2 family and does not dimerize with CD94, rather it is expressed as homodimer and associates with the DAP10 adaptor protein (Wu et al. 1999).

A common structural feature of the inhibitory receptors is the presence of cytoplasmic ITIM (I/V/L/SxYxxL/V sequences) that after tyrosine phosphorylation, recruit protein tyrosine phosphatases (SHP-1 and SHP-2) involved in the downregulation of NK cell activity (Burshtyn et al. 1996; Campbell et al. 1996; Fry et al. 1996; Olcese et al. 1996). CD94/NKG2A/-B complex contain cytoplasmic ITIMs and are generally considered to be inhibitory receptors. CD94/NKG2C and CD94/NKG2E/-H are homologous to NKG2A/-B but lack ITIMs, possess a charged amino acid in the transmembrane region and exert activating functions.

2.2 Ligands for CD94/NKG2 receptors

Moretta and co-workers, in 1994 proposed that the CD94 receptor could recognize certain HLA molecules on the target cell. Moreover, the hypothesis that the CD94/NKG2 C-type lectin receptor complex may be involved in NK cell mediated recognition of different HLA allotypes was further supported by the functional data reported by Sivori et al. 1996 and Phillips et al. 1996. However, although NK cells expressing these C-type lectin receptors could recognize in a CD94 dependent way, target cells expressing HLA-A, HLA-B, HLA-C or HLA-G, it was not possible to demonstrate a direct interaction between these ligands and the receptors. Subsequent experiments revealed that the true ligand bound by the CD94/NKG2A/B as well as the CD94/NKG2C complex was HLA-E, a non-classical MHC class I molecule, that binds leader peptides derived from the signal sequences of classical HLA-A, HLA-B, HLA-C or HLA-G alleles (Borrego et al. 1998[(1)(2)]; Braud et al. 1998[(1)]; Lanier et al. 1998; Lee et al. 1998[(2)]; Brooks et al. 1999). Since HLA-

E expression depends on the availability of classical MHC class I leader peptides, downregulation of MHC class I expression consequently leads to a reduction of HLA-E expression at the cell surface. Accordingly, although indirect, the interaction between CD94/NKG2 and HLA-E is one of the central mechanisms by which NK cells monitor the overall expression level of multiple MHC class I molecules.

2.2.1 HLA-E molecule

Major histocompatibility class I molecules (MHC-I) are classified into two groups; classical MHC-I or class Ia proteins (HLA-A, HLA-B and HLA-C) which are encoded by highly polymorphic genes and are expressed in most nucleated cells, and non-classical MHC-I or class Ib molecules (HLA-E, HLA-F and HLA-G), encoded by genes which are much less polymorphic and often have restricted patterns of expression. In humans, the best understood of these class Ib antigens is human leukocyte antigen (HLA)-E. Unlike the other MHC class Ib molecules, HLA-E is transcribed virtually in all human tissues and cell lines, although at lower levels than MHC class Ia antigens (Wei et al. 1990; Braud et al. 1997). The gene encoding HLA-E is located between HLA-A and HLA-C on the short arm of chromosome 6. With only nine alleles encoding three different proteins, HLA-E is the least polymorphic of all MHC class I molecules. Two HLA-E molecules, encoded by HLA-E*01:01 and HLA-E*01:03, exist with about equal frequencies in the human population (Geraghty et al. 1992; Strong et al. 2003; Antoun et al. 2009). The two confirmed alleles, HLA-E*01:01 and HLA-E*01:03 have been referred as HLA-E^R and HLA-E^G respectively since they differ in one amino acid, having either arginine (HLA-E*01:01) or glycine (HLA-E*01:03) located at position 107 on the α2 domain of the HLA-E heavy chain (Grimsley et al. 2002). Recently, a novel HLA-E allele, HLA-E*01:03:05 was identified in two Brazilian individuals. This novel HLA-E variant has a synonymous substitution with a G to C transversion at the position 1625, third base of codon 189 in exon 4 (Veiga-Castelli et al. 2012). The extremely high degree of conservation between species at the HLA-E locus, especially in the peptide-binding region (Connolly et al. 1993; Boyson et al. 1995) is consistent with an important, conserved, biological function of the peptide-HLA-E complex (Aldrich et al. 1994; Braud et al. 1997).

Like other MHC class I molecules, HLA-E is a heterodimer consisting of a α heavy chain, a type I integral membrane glycoprotein and a light chain, β-2 microglobulin (β2m) which is a soluble protein (Adams et al. 2013). The extracellular region of the heavy chain folds into three domains (α1, α2 and α3), with β2m contributing a fourth domain. As in MHC class Ia molecules, the α1 and α2 domains form the peptide-binding site: a groove on the upper surface of the MHC class I molecule, which bind antigenic peptides. However, this peptide binding groove is highly specialized to mediate an exquisitely specific association with 9 amino-acid long peptides derived from MHC class I signal sequences (Braud et al. 1997; O´Callaghan et al. 1998[(1)(2)]).

2.2.2 Processing of MHC class I molecules. Generation of HLA-E epitopes.

Unlike classical MHC class I molecules, HLA-E associates with a narrow set of nonameric peptides, mostly derived from the leader sequences of classical MHC class Ia molecules. These signal peptides are encoded by the N-terminal region of MHC class I molecules, which serve as

hydrophobic segments to target the newly synthesized proteins to the endoplasmatic reticulum (ER). Once in the ER, the N-terminal signal peptide is released from the class I protein by the signal peptidase (SPase) (Martoglio et al. 1998). The native class I protein is chaperoned by calnexin and incorporated into a protein complex responsible for peptide loading, the so-called peptide loading complex (PLC) (Cresswell et al. 2005). The cleaved signal peptide of MHC I molecule, usually 24 amino acids long, remains in the ER membrane due to its hydrophobicity and needs a second cleavage to be liberated from the membrane. This second cleavage is mediated by the signal peptide peptidase (SSPase), an aspartic protease, which acts within the ER membrane (Lemberg et al. 2001). SSpase mediated cleavage results in release of the epitope-containing leader peptide to the cytosol. This leader peptide gets rewarded with a second life becoming a peptide, which could be presented by an HLA-E molecule. To be loaded onto the HLA-E molecule, the precursor peptide is further shaped by the proteasome to make it suitable for transport back into the ER, a function performed by the transporter associated with antigen processing (TAP) (Bland et al. 2003). ER resident chaperones facilitate the folding of the HLA-E molecule heavy chain and β2m, which bind TAP in a complex where tapasin plays a critical role, bridging HLA-E and TAP. Tapasin is also required to facilitate the binding and loading of the high affinity peptide to the HLA-E molecule (Ulbrecht et al. 1998; Braud et al. 1998[2]; Lee et al. 1998[1]). After peptide loading, MHC class I molecules dissociate from TAP and cluster at the export site on the ER membrane where they are selectively recruited into cargo vesicles for transport to the Golgi apparatus. HLA-E molecules then traffic through the Golgi apparatus to the plasma membrane where it can serve as a ligand for particular receptors.

2.2.3 Peptides presented by HLA-E molecule.

The peptide-binding groove of HLA-E has similar dimensions to that of the classical MHC class I molecules, but its surface features are distinctive. The groove is highly hydrophobic, which favours binding of hydrophobic leader peptides. Pockets along the length of the groove are well adapted to bind specific amino acids. Three hydrophobic pockets at peptide position 1, 7 and 9 bind methionine, valine and leucine respectively and two pockets at positions 3 and 6, which bind alanine and threonine respectively. A number of hydrophobic interactions, as well as hydrogen bonds, between residues in the groove and atoms of the peptide chain, occur along the length of the peptide ligand. For these, and other specific interactions to occur the peptide needs to have a correct conformation and length. This restricts the ability of peptides to bind and stabilize the HLA-E molecule.

A standard leader sequence is well conserved throughout MHC class I molecules, and is of the form MAVMAPRTVLLLSGALALTQTWA. The first structure of HLA-E in a complex with the leader sequence peptide from HLA-B*08 (VMAPRTVLL) was described by O´Callaghan et al. 1998. This study demonstrated that leader peptide specificity is a highly developed property of HLA-E. Due to the extensive repertoire of human HLA-class I molecules, the exact sequence of the leader peptide especially at the positions 2, 7 and 8 differs, but most of them bind to HLA-E efficiently with the exception of the leader peptides from some HLA-B alleles, which carry a

threonine at position 2 of the peptide, as well as the leader peptide derived from the HLA-E itself (Lee et al. 1998[2]; Stevens et al. 2001; Strong et al. 2003).

Although, HLA-E is normally found associated with a limited peptide repertoire, other lines of evidence have revealed that peptides from proteins other than MHC class I molecules can bind HLA-E. These peptides include the VMAPRTLVL and the VMAPRTLIL peptides derived from the UL40 glycoprotein leader sequences of two different human cytomegalovirus (CMV) strains (Toledo and AD169), matching exactly the leader sequence peptides of various HLA-A and –C alleles (VMAPRALVL and VMAPRTLIL respectively) (Heatley et al. 2013). Importantly, unlike the MHC class I leader peptides, the gpUL40-derived peptides are assembled on HLA-E in a TAP-independent manner (Tomasec et al. 2000). Peptides derived from the heat shock protein 60 (hsp60, QMRPVSRVL) (Michaëlsson et al. 2002), the ATP-binding cassette transporter multidrug resistance-associated protein 7 the (MRP7, ALALVRMLI) (Wooden et al. 2005), the *Salmonella enterica* serovar TyphiGroEL protein (GroEL, KMLRGVNVL) (Salerno-Goncalves et al. 2004), the human immunodeficiency virus (HIV) gag protein (HIVp24, AISPRTLNA) (Naterrmann et al. 2005[1]), and gliadin (gliadin α2 chain, SQQPYLQLQ) (Terrazzano et al. 2007) have also been shown to bind to the HLA-E molecule. Recently, multiple peptides derived from *Mycobacterium tuberculosis* have been described as having the capacity to bind and be presented by HLA-E (Joosten et al. 2010).

The binding of viral protein peptides, as is typical for classical MHC class Ia molecules, has also been demonstrated for HLA-E and examples of these include peptides from the influenza matrix protein (InflM, ILGFVFTLT) (Ulbrecht et al. 1998), Epstein-Barr virus (EBV) BZLF-1 protein (BZLF-1, SQAPLPCVL) (Jorgensen et al. 2012) and the Hepatitis C virus (HCV) core protein (HCV Core, YLLLPRRGPRL) (Nattermann et al. 2005[2]; Orr et al. 2010; Cheent et al. 2013). Interestingly, two alternative splicing isoforms of mutant peroxiredoxin 5 (Prdx5) encode two nonapeptides Prdx5 splice variant (Prdx5Δ2, AMAPIKTHL) and Prdx5 splice variant (Prdx5Δ2, 3, AMAPIKVRL) also able to bind HLA-E (Sensi et al. 2009).

2.3 Presentation of HLA-E-bound peptides to CD94/NKG2 receptors and receptor response

The identification of HLA-E as a ligand for CD94/NKG2 receptors was achieved essentially simultaneously by several groups. Two groups showed that stabilization of HLA-E expression at the cell surface by incubation with synthetic peptides derived from the signal sequences of a number of HLA class I molecules led to protection of these peptide-treated cells from NK cell lysis. Further, treatment with CD94-specific but not killer cell inhibitory receptor (KIR)-specific mAbs could block this HLA-E-mediated protection of target cells (Borrego et al. 1998; Lee et al. 1998[2]). In contrast, the experiments of Braud et al. depended on the utilization of new method for identifying antigen specific T cells; peptide-HLA tetrameric complexes, a technique developed by Mark Davis´ laboratory in 1996 (Altman et al. 1996; O´Callaghan et al. 1999; McMichael et al. 1998). Tetramers of HLA-E, loaded with MHC I leader peptides, bound to a significant population of peripheral blood lymphocytes expressing CD56 and CD16, both of which are markers for NK cells (Braud et al. 1998). The identification of mAbs able to block the binding of the HLA-E tetramers to NK cell surface molecules led to the identification of the heterodimeric

CD94/NKG2A, as well as NKG2B or -C surface molecules. These proteins were specific and selective receptors for HLA-E, that could trigger inhibitory (CD94/NKG2A-B receptor) or activating (CD94/NKG2C receptor) signals in the NK cells. The finding that CD94/NKG2 receptors can recognize and directly bind HLA-E has been later confirmed by other groups (Llano et al. 1998; Vales-Gomez et al. 1999; Brooks et al. 1999).

Based on the structure of MICA bound to NKG2D, Li and coworkers (Li et al. 2001[2]) proposed that CD94/NKG2A would bind HLA-E with CD94 oriented over the α1 helix of HLA-E and C-terminal part of the peptide, and NKG2A over the α2 helix. Subsequent binding and mutagenesis studies of HLA-E helices (Kaiser et al. 2005; Sullivan et al. 2007; Wada et al. 2004) coupled with the resolution of the structure of the non ligated CD94/NKG2A heterodimer (Sullivan et al. 2007) provided further support for this orientation, indicating that CD94 is likely responsible for reading out the C terminus of the peptide and that the two most critical peptide contact points for CD94/NKG2 are positions P5, an invariant Arg, and P8, a hydrophobic residue that varies between class I leader peptides (Miller et al. 2003; Sullivan et al. 2007). Finally, the solution of the crystal structure of the complex between CD94/NKG2A and HLA-E (Kaiser et al. 2008) has provided detailed insight into the recognition mechanisms of this family of immunoreceptors, accounting for the affinity differences between NKG2A and NKG2C for HLA-E and between different peptides for NKG2x receptors. Sequence differences in NKG2x residues 167-170, which form part of the heterodimer interface, account for the affinity difference between CD94/NKG2A and CD94/NKG2C for given HLA-E-peptide complexes.

Several proteins other than MHC class I leader peptides have also been shown to encode peptides that can bind to HLA-E (Nattermann et al. 2005[2]; Ulbrecht et al. 2000; Wooden et al. 2005). Taking into account the particular differences of these peptides from canonical MHC class I leader peptides, it seems reasonable to assume that CD94/NKG2x complex will interact with HLA-E with a reduced affinity but this remains to be confirmed.

In addition to being a ligand for the CD94/NKG2 receptors, HLA-E can also present peptides for recognition by $CD8^+$ T cells expressing conventional $\alpha\beta$TCRs (Heinzel et al. 2002; Pietra et al. 2003; Romagnani et al. 2004; Salerno-Concalves et al. 2004). The footprint of an HLA-E restricted TCR on HLA-E closely overlaps that of CD94/NKG2A on HLA-E (Sullivan et al. 2007; Petrie et al. 2008).

2.3.1 Affinity

There is a precise correlation between the nature of the peptide bound by HLA-E and immune recognition for function (Llano et al. 1998; Brooks et al. 1999; Vales-Gomez et al. 1999). In cellular systems, Llano and coworkers confirmed that there is a good correlation between the presence of a leader peptide with methionine at position 2 and recognition by CD94/NKG2A receptors, while Brooks et al. used recombinant receptor protein to examine the interaction of HLA-E with CD94/NKG2A and observed that at 26°C HLA-E could reach the surface without peptide and that peptides with threonine at position 2 could also promote surface expression of HLA-E, although with less efficiency than those peptides with a methionine at the same position. At 37°C, the stability of the HLA-E complex containing peptides with a threonine at position 2 was markedly reduced and these complexes rapidly disappeared from the cell

surface when cells were warmed from 26 to 37°C. The efficiency of receptor binding was shown to be similar in both cases. These workers also showed that the presence of an alanine at position 6 in HLA-Cw*0702, slightly reduced the stability of HLA-E at the cell surface and markedly reduced the binding of the receptor to HLA-E. Again in 1999, Vales-Gomez et al. using an *in vitro* system allowing direct measurement of the binding of soluble HLA-E to soluble CD94/NKG2 receptors, addressed the importance of the peptide bound to HLA-E for interaction with the CD94/NKG2A and CD94/NKG2C receptors. These data confirmed that leader peptides with threonine at P2 could refold HLA-E, but that these complexes were less stable than those where HLA-E was refolded using peptides with methionine in this position and these workers were the first to show that CD94/NKG2A – HLA-E-peptide interactions had kinetic parameters similar to those of the p58 KIR binding to HLA-C (Vales-Gomez et al. 1998). Moreover, the activating receptor (CD94/NKG2C) was found to bind to the peptide MHC complex with a much lower affinity than the inhibitory receptor (CD94/NKG2A) and that the major factor for the final outcome of the interaction between HLA-E and the CD94/NKG2-A/-C receptors is the nature of the bound peptide. Changes in the peptide sequence at residues P6 and P8, at least, may directly influence binding to CD94/NKG2, whereas substitutions at P2, P3 and P7 are more likely to affect binding indirectly by influencing peptide binding to MHC.

2.3.2 CD94/NKG2A-C ambivalence

NK cells form two distinct types of immunological synapse: a cytotoxic synapse (cNKIS) resulting in cell killing, and NK activation for cytokine and granule release, and an inhibitory synapse (iNKIS) that suppresses these functions. The nature and molecular composition of these synapses are very different (Bromley et al. 2001; Davies et al. 2001; McCann et al. 2002; Vyas et al. 2002). The formation and aggregation of activating receptors in a cNKIS requires an intact cytoskeleton and ATP (Lou et al. 2000; Bromley et al. 2001; McCann et al. 2002; Vyas et al. 2001; Vyas et al. 2002; Davis et al. 2004) whereas an iNKIS, polarizes to the site of contact in a cytoskeleton independent fashion (Fassett et al. 2001; Masilamani et al. 2006). Other data have shown that the majority of the CD94/NKG2A molecules exist as a membrane-mobile fraction and move freely within the plasma membrane (Lippincott-Schwartz et al. 2001; Sanni et al. 2004). However, after ligation, CD94/NKG2A is enriched at the site of the contact with the target and becomes markedly immobile (Sanni et al. 2004).

The ability of CD94/NKG2A receptor and its splice variant CD94/NKG2B to transmit inhibitory signals depends on the two ITIMs in the cytoplasmic tail of NKG2A and -B. After interaction with the ligand, the tyrosine residue in each ITIM becomes phosphorylated thereby facilitating the binding and activation of the SHP-1 and SHP-2 tyrosine phosphatases, which lead to the suppression of NK cell activation (Kabat et al. 2002; Le Drean et al. 1998). These receptors inhibit activation signals by interfering with the actin cytoskeleton-dependent recruitment of ligated activating receptors into lipid rafts at the target contact sites (Stebbins et al. 2003; Watzl et al. 2003; Masilamani et al. 2006) and phosphorylated Vav1 has been described as a major target for the SHP-1 phosphatase (Stebbins et al. 2003; Masilamani et al. 2006).

In general, the inhibitory effect generated by CD94/NKG2A ligation can override the effect of activating signals, thereby preventing the killing of target cells and cytokine release. However, if the activation signals are very strong, CD94/NKG2A inhibitory signalling can be overridden (McMahon et al. 2002; Lanier et al. 2005; Ortaldo et al. 2005). Importantly, these effects are topographically restricted so that NK cells can simultaneously form both activating (aNKIS) and inhibitory synapses (iNKIS) with a susceptible or a resistant target cell and indeed lyse only the susceptible target but spare the resistant target respectively (Eriksson et al. 1999).

CD94/NKG2C-E-H activating receptors lack intrinsic sequences required for cellular activation. However, these receptors associate with the DAP12 adaptor protein that has ITAMs and can transmit activation signals upon ligand binding (Bellón et al. 1999; Lanier et al. 1998). Moreover, association of these receptors with DAP12 significantly enhances receptor cell surface expression levels (Lanier et al. 1998). In the CD94/NKG2C cNKIS, the kinase PYK-2, microtubule organizing center (MTOC) and paxillin translocate to the area of NK-target cell contact (Sancho et al. 2000). Further, it has been shown that the MAPK pathway of cellular activation is also involved in the CD94/NKG2C-mediated signalling (Carretero et al. 2000).

Under physiological conditions, all nucleated cells continuously express the HLA-E molecule, and ligation of its receptors leads to their down-regulation. A continuous supply of CD94/NKG2A receptors on the plasma membrane is maintained by the recycling of internalized receptors between the plasma membrane and endosomal compartment in a ligand independent manner (Borrego et al. 2002; Mellman et al. 1996). Although not much is known about intracellular CD94/NKG2C trafficking, it can be distinguished from that of CD94/NKG2A by its sensitivity to brefeldin A treatment (Borrego et al. 2002).

2.3.3 NKG2C: ¨Memory¨/¨memory-like¨ marker on NK cells?

Guma et al. 2004 published the very first data indicating that HCMV infection, but not other herpesvirus infections [Epstein-Barr virus (EBV), and herpes simplex virus (HSV)] could shape the NKR repertoire of healthy individuals by selectively promoting an expansion of CD94/NKG2C$^+$ NK and T cells. Later, co-culture of PBMCs from HCMV seropositive donors with HCMV infected fibroblasts was shown to produce a preferential expansion of CD94/NKG2C$^+$ NK cells by a mechanism that probably involved the interaction of the CD94/NKG2C receptor with the infected cell (Guma et al. 2006[(1)]). In studies with other viral infections, like Hantavirus, and Chikungunya virus, a similar expansion of NKG2C$^+$ NK cells was described (Bjorkstrom et al. 2011; Petitdemange et al. 2011), although an influence of previous exposure to HCMV infection could not be completely excluded in these patients. An increased frequency of NKG2C$^+$ NK cells was also detected in HCMV seropositive individuals infected with HIV (Brunetta et al. 2010) and Hepatitis B virus (HBV) (Beziat et al. 2012). Importantly, the HLA-E molecule, a known ligand for the CD94/NKG2C receptor has been shown to be upregulated in HCMV (Prodhomme et al. 2012), Hantavirus (Bjorkstrom et al. 2011) and HIV (Nattermann et al. 2005[(1)]) infections. It has been shown that NK cell education (ie, expression of a self-specific KIR) is required for the efficient expansion of NKG2C$^+$ NK cells, at least *in vitro* (Beziat et al. 2013). Moreover these *in vitro* data also support a model where the maintenance of the NKG2C$^+$ expansion over years depends on

proliferation and differentiation of $CD57^-$ NK cells, rather than the pre-existing differentiated $CD57^+$ NK cell subset. In some rare $HCMV^+$ but $NKG2C^-$ donors NK expansions involving educated NK cells expressing activating KIRs (KIR2DS4, KIR2DS2 and KIR3DS1) (Beziat et al. 2013), as well as receptor KIR2DL2/3 (Foley et al. 2011; Bjorkstrom et al. 2011; Petitdemange et al. 2011; Bezait et al. 2012) have been described.

In the setting of solid-organ transplantation, allogenic hematopoietic stem cell or umbilical cord blood transplantation HCMV reactivation results in the induction of functional $CD57^+$ and $NKG2C^+$ NK cells (Della et al. 2011; Foley et al. 2012; Lopes-Verges et al. 2011). In humans, CD57 is expressed preferentially by a subset of NK cells with a highly mature phenotype (Bezlat et al. 2010; Bjorkstrom et al. 2010; Lopez-Vergez et al. 2010) suggesting that CD57 might mark NK cells that have been clonally expanded by infections. Interestingly, the expanded $NKG2C^+$ cells did not just express the CD57 marker, but were also more potent producers of IFN-γ than their $NKG2C^-$ counterparts. Similarly, another study demonstrated that after hematopoietic cell transplantation, $NKG2C^+$ NK cells from CMV-positive donors expanded only in CMV^+ recipients, whereas $NKG2C^+$ NK cells from CMV-negative donors did not, consistent with the existence of a secondary response against HCMV (Bjorkstom et al. 2011). However expansions of $NKG2C^+$ lymphocytes do not always correlate with HCMV infection since the frequencies of $CD94/NKG2C^+$ peripheral blood NK and T cells are increased in patients during the acute phase of Steven Johnson syndrome (SJS) and Toxic epidermal necrolysis (TEN) and these cells can degranulate in response to $HLA\text{-}E^+$ cells in an NKG2C-dependent manner (Morel et al. 2010).

In summary, all of these data suggest that the NKG2C receptor is actively participating in the immune response against HCMV. However, further investigations are needed to fully define how NKG2C participates in processes underlying the generation of NK memory. It is important to note that although recently it has been suggested that human NK cells can exhibit a memory-like response, at present this has not been confirmed definitively. Moreover, it is still not entirely clear whether the phenomenon of NK memory depends on antigen driven expansion of NK cell subpopulations, or whether these cells are a variant of human cytokine-induced memory like NK cells (Cooper et al. 2009; Lee et al. 2012; Ni et al. 2012; Romee et al. 2012).

2.3.4 Non-classical MHC class I restricted T cells

Under usual homeostatic conditions the peptide repertoire bound to HLA-E is highly restricted to self, MHC derived signal sequences. However, under cellular stress conditions such as malignant cell transformation or intracellular infection with chronic pathogens, a new peptide repertoire can be loaded onto HLA-E molecules and presented to $CD8^+$ T cells (CTLs) able to kill tumour cells or infected cells. Thus, while HLA-E was first described as a ligand for CD94/NKG2A (inhibitory) and CD94/NKG2C (activating) NK cell receptors, it is now clear that it can also present peptides for $\alpha\beta$ TCR-mediated recognition (Li et al 2001[(1)]; Pietra et al. 2001; Garcia et al. 2002; Heinzel et al. 2002). Several observations have also raised the possibility that activation-induced expression of CD94/NKG2 on a subset of CTLs may critically influence CTL function (Mingari et al. 1998[(2)]). Specifically, cross-linking of KIRs or CD94/NKG2A receptors expressed on T cells strongly inhibited CTL-mediated cytotoxicity (Ferrini et al. 1994; Mingari et al. 1995; Phillips et al.

1995) indicating that these inhibitory MHC-recognizing receptors could contribute to the down-regulation of a CTL response.

A number of examples of HLA-E dependent presentation of bacterial and viral peptide antigens to human αβ TCR $CD8^+$ T cells have been documented. Several pathogens known to encode HLA-E binding peptides are: *Mycobacterium tuberculosis* (Mtb), *Salmonella enterica, Listeria monocytogenes,* CMV, HIV, HBV, EBV and HCV (Rodgers et al. 2005).

Mycobacterium tuberculosis (Mtb) represents a leading cause of infectious disease morbidity and mortality worldwide. Both, $CD4^+$ and $CD8^+$ T lymphocytes appear to be important for immune defence against mycobacterial infection. The importance of HLA-E restricted T cells in the host cell response to Mtb infection was first described in 2002 (Heinzel et al. 2002). These data represent the first demonstration of CTL recognition of a pathogen-derived antigen being presented in the context of HLA-E. Presentation of the Mtb-derived peptide was shown to be TAP independent and to require proteasomal processing. Another finding shows that mycobacteria reside in phagosomes of antigen presenting cells (APC), especially macrophages, and that these phagosomes enriched in HLA-E probably facilitates presentation of mycobacterial peptides to human HLA-E (Grotzke et al. 2009). Recently, a number of HLA-E restricted peptide epitopes from Mtb recognized by $CD8^+$ T cells have been identified. In this study *in silico* analyses of the Mtb genome predicted candidate HLA-E epitopes for recognition by human T cells (Joosten et al. 2010). Subsequently, a combination of HLA-E peptide binding studies and assays of the proliferation of human $CD8^+$ T cells from PPD-responsive adults and BCG-vaccinated infants established which of those peptides were true epitopes. The responding $CD8^+$ T cells had cytotoxic activity, and lysed the target cells in a peptide specific and HLA-E dependent manner, strongly suggesting HLA-E-peptide engagement by the TCR. However, these Mtb-reactive T cell lines also exhibited strong immunosuppressive properties, as they inhibited the proliferation of unrelated responder T cells, suggesting that this dual activity could be mediated by the same HLA-E-peptide induced T cells. Further studies need to be done to understand whether, HLA-E mediated antigen presentation in tuberculosis preferentially activates T cells with regulatory properties as compared to T cells with cytolytic activity. It is interesting to note that $CD8^+$ T cells specific for Hsp60sp bound to HLA-E play an important role in maintaining peripheral self-tolerance (Jiang et al. 2010) and also that murine $CD8^+$ T cells specific for the unconventional MHC class I molecule Qa-1 (the murine homolog of HLA-E) bound to peptides derived from the signal sequence of Hsp60 have also been reported to contribute to self/nonself discrimination (Sarantopoulos et al. 2004; Leavenworth et al. 2013).

In the context of the viral infections, one of the most common viruses infecting the human population is HCMV, causing a life-long persistent asymptomatic infection, which, however, may cause severe morbidity in immunocompromised individuals. HCMV has evolved an impressive variety of strategies to escape from recognition mediated by conventional (MHC class Ia-restricted) CTLs. Different CMV viral proteins are well known to inhibit MHC class I expression by the infected cells. Viral proteins, like US2 and US11 bind to nascent HLA class I chains to shuttle them from the ER to the cytosol, where they undergo proteasome-dependent degradation. The viral US3 protein binds HLA molecules and retains them in the ER while US6

binds TAP and blocks TAP-mediated transport of antigenic peptides to the ER. As a consequence, surface expression of MHC class I molecules, presenting possible viral proteins is down regulated, leading to inefficient antigen presentation to CTLs and viral escape from the adaptive immune system. On the other hand, human CMV itself, through the expression of gpUL40 protein, can supply peptides that can bind and be presented by HLA-E at the cell surface in a TAP independent manner (Ulbrecht et al. 2003; Hoare et a. 2006). This augmented surface expression of HLA-E via interaction with the inhibitory CD94/NKG2A receptor will allow escape from NK-cell attack. Interestingly, Pietra and coworkers in 2003, provided evidence that some human $CD8^+$ $TCR\alpha\beta^+$ T cells can recognize HLA-E when loaded with peptides derived from HCMV. These HLA-E restricted $CD8^+$ T cells could represent an additional type of effector cells playing a role in defence against a virus, which has evolved to escape recognition by both adaptive and innate immunity. Indeed, these HLA-E restricted $CD8^+$ T cells are capable of prompt production of IFN-γ and can kill infected cells (Mazzarino et al. 2005). The UL40 protein of HCMV, that supplies the viral peptides loaded onto HLA-E, is variable and strikingly, the ability of individuals to develop effective HLA-E restricted $CD8^+$ T cell defence against CMV infection depends on the genotypes of the infecting HCMV strain as well as the HLA-A and HLA-Cw haplotype of the infected individual.

That HLA-E can play an important role in regulating antiviral immunity has also been shown for hepatitis C virus (HCV). Infection with this virus is associated with enhanced intrahepatic HLA-E expression (Nattermann et al. 2005[(2)]) where HCV gives rise to a peptide, corresponding to amino acids 35-44 of the core protein, that binds HLA-E, to stabilise and promote surface expression where it protects cells from NK cell mediated cytotoxicity. Apart from its interaction with NK cell receptors, it has also been demonstrated that peptide loaded HLA-E molecules could also be recognized by $CD8^+$ T cells via their TCR (Schulte et al. 2009). Schulte and coworkers also provided the first evidence for the different functional role of the two allelic variants ($HLA\text{-}E^R$ and $HLA\text{-}E^G$) of HLA-E in HCV infection.

HLA-E expression by tumour cells has been recently reported in several types of human cancers. For example, HLA-E is overexpressed in fresh lymphoma (Marin et al. 2003), ovarian carcinomas (Malmberg et al. 2002), gliomas (Wischhusen et al. 2005), melanomas (Derre et al. 2006) and colon cancer (Bianchini et al. 2006). Expression of HLA-E at the cell surface, that can bind inhibitory CD94/NKG2A receptor expressed by NK and T cells, would seem to favour tumour cells escape from the immune system. To date, the potential role of HLA-E as an antigen-presenting molecule for tumour specific $CD8^+$ T cells is poorly understood, but in murine models it has been shown that Qa-1b, a nonclassical major histocompatibility complex molecule that binds MHC class I derived leader peptides and functions as a ligand for germ line receptors CD94/NKG2, dominates the immune response to tumours with defects in antigen processing (Oliveira et al. 2010). The basis of this phenomenon seems to be that deficiencies in the normal processing pathway leads to the replacement of the normally loaded HLA leader peptides by a novel peptide repertoire that contains immunogenic neoantigens for $CD8^+$ T cells. Thus, in processing-deficient tumour cells, the absence of HLA-leader peptides relieves the inhibition

through CD94/NKG2A while presentation of a novel repertoire of immunogenic peptides, stimulates cytotoxic $CD8^+$ T cells.

Interestingly, peptides derived from alternative splicing events of the peroxiredoxin5 (Prdx5) gene can be presented by HLA-E (Sensi et al. 2009). Increased levels of Prdx5 can be detected in several diseases, such as osteoarthritis and tendon degeneration as well as in melanoma cells as a result of different forms of cellular stress. Although no evidence for immune recognition of the Prdx5 peptide-HLA-E complex was shown, perhaps the endogenous and environmental stress conditions alter the normal cellular processes and lead to cellular damage that could contribute to HLA-E stabilization leading to $CD8^+$ mediated recognition and elimination of tumour-stressed cells. In contrast, recent analyses of ovarian and cervical cancer samples have indicated that the HLA-E molecule is frequently over expressed in these two tumour types. *In situ* analyses of inhibitory CD94/NKG2A and activating CD94/NKG2C expression, revealed a frequent expression of the inhibitory receptor on intraepithelial $CD8^+$ T cells, whereas NK cells, the predominant cell type expressing these two receptors, were hardly found in both tumour types. Importantly, the beneficial prognostic effect associated with the presence of high numbers of infiltrating CTLs in ovarian cancer was completely neutralized by the strong expression of HLA-E, indicating that CTL infiltration in this type of cancer is associated with better survival only when HLA-E expression is low and that intratumoural CTLs are inhibited by the CD94/NKG2A receptor expressed on their surface (Gooden et al. 2011).

2.3.5 CD94/NKG2 receptors modulate antigen-specific T cell activity

CD94/NKG2 receptors, as mentioned previously, are expressed by small subpopulations of $\alpha\beta$ and $\gamma\delta$ T cells as well as by NK cells. About 5 % of peripheral human $CD8^+$ T cells express the inhibitory CD94/NKG2A receptor and interestingly, $CD94/NKG2^+\alpha\beta CD8^+$ T cells are oligoclonal populations characterized by an effector memory phenotype (McMahon et al. 2001). Generally, the NK receptors expressed by CTLs are characterized by their capacity to modulate, positively or negatively, TCR signalling but T cell activation remains under the control of the TCR (the TCR primacy rule) (Jabri et al. 2006). However some important exceptions to this rule have been described. For example during CMV infection, CTLs express surface NKG2C and similar observations have been made in conditions of chronic T cell inflammation. While these T cells could benefit the host in the case of infection, $NKG2C^+$ T cells have been suggested to be detrimental in chronic inflammation and may contribute to severe immunopathology, as suggested in celiac disease.

Transcripts for NKG2E and NKG2H have been also detected in primary human CTLs (Jabri et al. 2002), but their expression remains to be confirmed by specific antibodies. Both, CD94/NKG2A and CD94/NKG2C recognize and bind HLA-E molecule. Because CD94/NKG2C is poorly expressed by CTLs, ligand induction should generally translate into an inhibitory signal in the normal target cell conditions. In the case of disease conditions such as CMV (Guma et al. 2004) and severe celiac disease CTLs (Meresse et al. 2006) can express CD94/NKG2C, but they do not express CD94/NKG2A, thus avoiding a conflict between these opposite forms of signalling.

TCR stimulation, in a combination with various cytokines including interleukin-15 (IL15), transforming growth factor-β (TGF-β), IL-2, IL-12 or IL-10, has been shown to up-regulate the surface expression of NKG2A on T cells (Mingari et al. 1998[(1)(2)]; Bertone et al. 1999; Derre et al. 2002), suggesting that NKG2A might be an activation induced event not dependent on TCR specificity. However, although the microenvironment might alter CD94/NKG2A surface expression, TCR interaction with cognate antigen seems to be a prerequisite for its induction *in vivo*. Importantly, IL-15, TGF-β, and IL-12 could induce CD94 but not NKG2A in the absence of concomitant TCR stimulation, further suggesting control of TCR over NKG2A expression. NKG2A may therefore participate in a negative feedback loop in which TCR stimulation up-regulates NKG2A and, in turn, NKG2A down-modulates TCR activation.

There is strong evidence indicating that CD94/NKG2 receptors on CTLs play essential roles in the control of these cells during important disease processes. The presence of abundant melanoma-specific CTLs in patients with progressive tumour has been correlated with expression of inhibitory NKG2A (Speiser et al. 1999), expansion of CD94/NKG2C/DAP12 expressing CTLs, as mentioned before, occurs in some pathological situations and ultimately, the loss of TCR control over proliferation might be involved in the malignant transformation of CTLs, as observed in celiac disease.

Objectives

The main objectives of the work presented in this thesis were:

1. Phenotypic and functional characterization of the orphan receptor CD94/NKG2H.

   1.1 Identify and characterize the cell types that express NKG2H protein.

   1.2 Determine the possible biological role of the $NKG2H^+$ cells in viral infections.

   1.3 To explore the function of NKG2H-expressing cells after specific stimulation of the receptor via monoclonal antibody

2. Identification and analysis of *Mycobacterium tuberculosis* specific T cell clones, using HLA-E tetramers loaded with specific peptide epitopes from mycobacteria, in the blood of patients with bladder cancer receiving BCG therapy.

Materials and methods

PBMCs.

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation. The blood was collected in a 50 ml falcon tube containing (1/1000) heparin (Sigma Aldrich), then diluted 1:2 with PBS, layered over an equal volume of Ficoll and centrifuged for 30 min at 1800 rpm without brake. PBMCs, accumulated at the interface between the plasma and the ficoll, were recovered, washed twice with PBS, and resuspended in RPMI supplemented with 10% human serum (HS)(Sigma Aldrich), 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 50 μM β-mercaptoethanol and incubated in a humidified incubator at 37^{o}C and 5% CO_2.

Cell lines, virus stocks and infections.

All cell lines were cultivated at 37^{o}C and 5% CO_2 in a humidified incubator and split when necessary. Human telomerase reverse transcriptase (hTERT) immortalized fibroblasts (HLA-type: HLA-A*01, B*08/*41, C*07/*17, DRB1*03/*07. DRB3^{+}, DRB4^{+}) were cultured in complete Dulbecco´s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin and 50 μM β-mercaptoethanol.

Stocks of HCMV strains Merlin, AD169, Toledo, TB/40 were prepared and titrated using standard methods (William J. Britt. 2010). In some experiments, preparations of *Herpes simplex* virus type 1 (HSV-1) or *Vaccinia* virus propagated and quantified as described previously (Earl et al. 2001; Blaho et al. 2005). hTERT fibroblasts were infected with HCMV, *HSV-1*, or *Vaccinia* in 24 well plates at 70% confluence in DMEM supplemented with 2% fetal bovine serum. The mock controls were not exposed to virus. After 18-20 hours of infection, the virus containing media was removed and cells were cultured in DMEM supplemented with 2% fetal bovine serum up to the point when used for the necessary experiment.

Lymphocyte co-cultures.

PBMCs were incubated in 24 well plates (1.5-2.10^{6}/well) in complete RPMI medium, supplemented with 5% fetal bovine serum and 5% human serum in the presence of mock or HCMV infected fibroblasts. Recombinant human IL-2 (50 U/ml) (Peprotech) was added to all cultures at day 3. Cells were then incubated for 3, 7 or 10 days. Some experiments were done in a Transwell (0.4μm) permeable support where PBMCs were incubated separately in the upper chamber, avoiding physical contact with mock or HCMV infected fibroblasts, cultured in the bottom chamber.

Flow cytometry.

For immunofluorescence staining of PBMCs, cells were washed with PBS containing 1% FBS, 0.5% BSA, and 0.05% sodium azide (S-PBS), pretreated with 1% normal human serum to block FcR, and subsequently stained with the fluorescently labeled and/or unlabeled mAb specified in each experiment, using the concentrations recommended by the manufacturer. After staining, the labelled cells were analysed by flow cytometry [Gallios (Beckman Coulter) or Cytomics FC500 (Beckman Coulter)]. All the staining was performed for 20 min on ice and in the dark, maintaining the cells at 4^{o}C until analysis.

For tetramer staining, 100 μl of heparinized blood were transferred to cytometry tubes. For all tetramers, a concentration of 0.5 μg per 100 μl of blood was used. The tetramer conjugated with streptavidin-PE was added and the sample was incubated for 20 min at room temperature before adding the mixture of mAbs. 1ml of lysing solution (VersaLyse, Beckman Coulter) in 2% paraformaldehyde (PFA) was added into the tubes and samples were vortexed, incubated for 30 min at room temperature and left at 4^{o}C for overnight (o/n). Samples were analysed on a Gallios (Beckman Coulter) flow cytometer and the acquisition was performed, gating on the lymphocyte population.

Flow cytometry data were analysed using either Kaluza v1.2 or FlowJo V9.6.2 cytometry analysis programs.

The following antibodies and reagents were used in phenotyping and functional assays:

Molecule	**Clone**	**Supplier**
CD3	CRL-8001	ATCC
CD3-FITC	UCHT1	BioLegend
CD3-PacificBlue	OKT3	BioLegend
CD4-APC	OKT4	BioLegend
CD8-PeCy7	RPA-T8	BioLegend
CD28	CD28.2	BD Biosciences
CD56-PE	MEM-188	BioLegend
CD56-PerCP/Cy5.5	N901	BeckmanCoulter
CD56-APC	CMSSB	eBioscience
CD69-APC/Cy7	FN50	BioLegend
CD161-Violet 421	HP-3G10	BioLegend
CD107a-APC	H4A3	BioLegend
Mouse IgG1	MOPC-21	Sigma
NKG2A-PE	Z199	BeckmanCoulter
NKG2C-PE	134591	R&D systems
NKG2H	633810	R&D systems
Annexin-V-FITC	731725	Beckman
7AAD	/	Beckman Coulter

RNA isolation, cDNA synthesis and Quantitative RT-PCR.

Total cellular RNA was extracted from PBMC cultured with mock or HCMV Merlin infected fibroblasts at day 0, day 3 and day 7 using RNA extraction kit (Qiagen). Reverse transcription to generate first strand cDNA was performed by standard protocols using random hexamers (Roche) and SuperScript II Reverse Transcriptase (Invitrogen) in the presence of RNase inhibitors (Promega).

cDNA was used as a template to quantify *nkg2h* gene expression using the following primers 5´-TGTGCAATGCTACATGTACGTG and 5´-TGATGCACTGCAAGCTCAAGC. qPCR reactions were carried using Hot FIRE Pol EvaGreen qPCR Mix Plus (ROX), (Solis BioDyne), in a final volume of 8μl. Reactions were run with Applied Biosystems 7900HT. Amplification was performed as follows: 50^{o}C for 10´, 95^{o}C for 2´, 40 cycles at 95^{o}C for 15´´and 60^{o}C for 1´.

Each experiment was performed in triplicates and repeated 3 times. The relative expression was calculated using the $2^{-(_{\Delta\Delta}Ct)}$ method using β actin for normalisation.

Degranulation assay.

Lysosome-Associated Membrane Protein 1 (LAMP-1, CD107a) is a protein present in the membrane of the multi-vesicular bodies that store lytic granules inside cytotoxic lymphocytes. Subsequent to cell activation, cytotoxic granules are released and LAMP-1 is transferred to the cell surface during the fusion of these lytic granules with the cell plasma membrane. Thus, degranulation can be measured by quantification of increases in surface LAMP-1 expression.

After a 7 day culture period of PBMCs with mock or HCMV infected fibroblasts, Monensin at 2 μM final concentration together with APC-labelled mAb against CD107a (Lamp1) at 3 μg/well was added to the media in all wells and incubated for an additional 5.30 h at 37^{o}C in 5% CO_2 supplemented incubator. Monensin prevents the acidification of endocytic vesicles avoiding the degradation of re-internalized LAMP-1 proteins from the surface and allowing the visualization of this marker following stimulation. After the incubation period, cells were surface stained as described previously for the CD3-FITC and CD56-PE markers. The percentage of single Lamp-1 positive or CD56/Lamp-1 and CD3/Lamp-1 double positive cells was determined by flow cytometry.

Stimulation of PBMC using immobilized antibody

To immobilize stimulating antibody, 24 well plates were pre-coated with purified human anti-CD3, anti-CD3/CD28 or anti-CD3/NKG2H mAbs in PBS and incubated for 2-3 hours at RT. The mAb concentration is specified in each experiment. Unbound mAb was removed by washing the plate once with PBS. PBMCs, at 1.5-2.10^{6} cells/well were added to the mAb coated plates in RPMI containing 10% HS and incubated for 48 or 72 hours at 37^{o}C and 5% CO_2 in a humidified incubator. After this period of incubation, cells were collected, washed with S-PBS, stained with CD3-Pacific Blue and CD69-APC/Cy7 mAbs and analysed by flow cytometry.

Proliferation assay.

CFSE (5-(and 6)-Carboxyfluorescein succcinimidyl ester) (Invitrogen) is a cell-permeable dye widely used to stain cells and monitor cell proliferation, both *in vivo* and *in vitro*. CFSE is a colourless and non-fluorescent dye that can easily cross the intact cell membrane. Once inside the cell, intracellular esterases cleave the acetate groups to yield the fluorescent carboxyfluorescein molecule which forms stable conjugates with membrane and intracellular proteins (Lyons et al. 1999). After each round of cell division the CFSE fluorescence intensity within daughter cells is halved, thus the number of rounds of cell division undergone by a cell can be followed by measuring the progressive halving of CFSE intensity.

PBMCs were resuspended in RPMI and incubated for 5-7 min at 37^{o}C in the incubator with the intracellular fluorescent dye CFSE at a final concentration of 2.5 μM. After two washes, CFSE labelled PBMCs were cultured in 24-well plates to which mAb had been pre-absorbed as described. CFSE labelled cells were further stained for surface markers and flow cytometry analyses were performed on days 2, 3 and 4 after stimulation.

Cell viability assay.

Loss of plasma membrane integrity is one of the earliest features changes detected in apoptotic cells, resulting in the translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the membrane. Annexin-V has a high affinity for PS, so it binds to cells with exposed PS, marking cells undergoing early apoptotic changes. At late stages of apoptosis or when necrosis occurs, the integrity of the plasma membrane is damaged. 7AAD is a dye that does not pass through an intact membrane, but can get into dead and damaged cells. Thus 7AAD stains cells that are in late apoptosis or necrotic.

Anti-CD3, anti-CD3/CD28 or anti-CD3/NKG2H stimulated PBMCs were collected, washed with S-PBS and surface stained with CD3-Pacific Blue and CD69-APC/Cy7 specific antibodies. Cells were washed with PBS and stained with Annexin-V FITC and 7AAD in annexin binding buffer (Invitrogen) following the instruction of the manufacture.

Antibody blocking assay

Purified PBMCs were cultured with immobilized anti-CD3 or anti-CD3/NKG2H antibody pre-coated plates as described above. 10 μg/ml of soluble, purified anti MHC class I antibody (HP-1F7, Perez-Villar et al. 1997) was added in RPMI containing 10% HS and incubated for 48 hours at 37^{o}C and 5% CO_2 in a humidified incubator.

For supernatant transfer experiments paired set of ¨donor¨ and ¨recipient¨ cell cultures were used. PBMCs from healthy donors were cultured in wells of plates pre-coated either with anti-CD3 or anti-CD3/NKG2H antibodies as described. After 48 hours of stimulation the supernatant from these cultures was collected and frozen at -20^{o}C. Later, the recipient cultures were prepared; PBMCs from healthy donor were purified and cultured in anti-CD3 or anti-

CD3/NKG2H antibody coated plates. Stored supernatant from the donor cultures was diluted 1:2 or 1:5 in complete RPMI medium supplemented with 10% human serum and then added to the corresponding recipient culture. PBMCs stimulated with immobilized anti-CD3 antibody in complete RPMI supplemented with 10% human serum were used as a control. Recipient cultures were maintained in a humidified incubator at 37^{o}C and 5% CO_2 for 48h until they were harvested and stained for the surface CD69 activation marker. The percentage of positive cells was analysed by flow cytometry.

Preparation of constructs for the expression of soluble CD94, NKG2H and HLA-E proteins.

PCR reactions were performed using standard protocols in a final reaction volume of 50 μl under similar reaction conditions employing *Pwo* (Roche) or *Taq* (Biotools/Roche) polymerase. 4% DMSO was used for some reactions to increase the PCR efficiency. For some amplification Mg^{2+} titration was also carried out, over a range of 0.5 mM to 2.5 mM in steps of 0.5 mM.

The amplification conditions used for the different sets of primers were: an initial denaturation step of 4΄30΄΄ at 95^{o}C followed by 25/35 cycles of denaturation (95^{o}C for 30΄΄), primer annealing (55-58^{o}C for 30΄΄), and extension (68^{o}C or 72^{o}C for 2΄), followed by a final incubation at either 68^{o}C (*Pwo*) or 72^{o}C (*Taq*) for 7΄ to allow for complete extension. PCR products were electrophoresed on 1-2% agarose and stained with ethidium bromide for visualization.

The primers used in all amplification reactions were:

Product	**Forward primer**	**Reverse primer**
sHLA-E	5΄CATGCCATGGGTTCTCATTCTTTAAAATATTTTCATACTTCTGTATCTCGTCCCGGCCG	5΄CCCAAGCTTATTATTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCAGACCGCCACCCGGCTTCCATCTCAGGGTGACGGGCTC
sNKG2H	5΄TTCATGAGCGGCCTGAACGACATCTTCGAGGCTCAGAAAATCGAATGGCACGAACATATGGAGCAGAACAATTCTTCCCCGAATGCAAGA	5΄CGGGGATCCTTATGCAATCATAATATCATTTCTG
sCD94	5΄CGCGGATCCGCCACCATGGCAGTGTTTAAGACC	5΄TTTTCCTTTTGCGGCCGCTTAAATGAGCTGTTGCTTACAGAT

Patients and healthy donors.

A total of 9 patients (7 males and 2 females), mean age 73 years (61-85 years), diagnosed with non-muscle invasive bladder cancer (NMIBC) were available for a pilot study. All patients analysed here received intravesical instillations of BCG, starting with a weekly instillation for six weeks followed by a rest period of two months and two weekly instillations during three years (Table 1 and Scheme 1). Blood was obtained from patients before each BCG instillation.

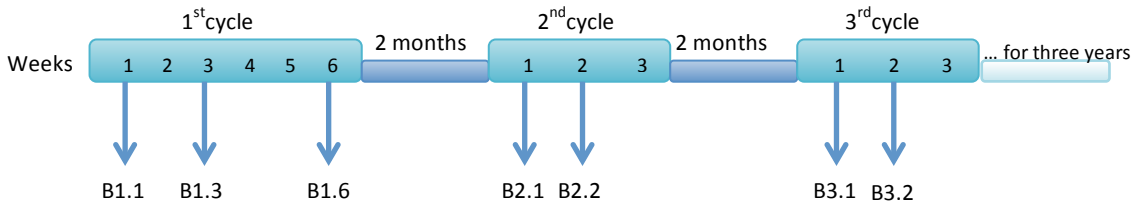
Informed consent was obtained from all the patients and the study was approved by the local and regional ethical committees (CEIC Hospital Infanta Sofía, CEIC Hospital La Paz and CEI CSIC)

Blood samples from a total of 19 healthy volunteers, 11 female and 8 male, with median age of 36 years, were also obtained during the study. All subjects had no known chronic/acute infections or inflammatory diseases for at least 1 month preceding the blood sampling. All of the anonymous volunteers agreed and gave written consent to the study. Samples of 30 ml venous heparinized blood were obtained from all subjects.

Table 1. Patient characteristics with tumour stage and grade.

No	Gender	Age	Tumour characteristics*	Type of instillation**
1	M	70	T1G3	BCG 81
2	M	70	TaG3	OncoTICE
3	M	61	T1G3	BCG 81
4	M	81	TaG3+Cis multiple	OncoTICE
5	F	85	T1G3	OncoTICE
6	M	73	Ca in situ	BCG 81
7	M	81	Cis	BCG 81
8	F	81	T1G3	BCG 81
9	M	71	T1G3	OncoTICE

* Ta/T1 indicate papillary tumors. Cis: carcinoma in situ
** BCG: Bacillus Calmette-Guerin; BCG 81/OncoTICE: BCG strains



Scheme 1: BCG treatment schedule in bladder cancer patients. Sample collection was done at first, third and last instillation of the first cycle and before the first and second instillation in every following cycle.

HLA-E tetramer production.

HLA-E constructs for expression of soluble molecules were prepared, as described above, using as template a plasmid encoding the extracellular domains of HLA-E (Vales-Gomez et al. 1999). A sequence recognized by the biotinylation enzyme BirA (AviTag) was introduced at the C-terminus by PCR. The construct also introduced modifications at the N-terminus to reduce the GC content and increase HLA-E expression yield in BL21 DE3 bacteria. This construct of HLA-E (891bp) was sub-cloned into pGMT7 vector (Reid et al. 1996; Cull and Schatz, 2000).

HLA-E tetrameric complexes were generated as described (Garboczi et al. 1992; Altman et al. 1996; Fan et al. 1996; O´Callaghan et al 1998[(2)]; Vales-Gomez et al. 1999). HLA-E and β2m proteins (Garbocziet al. 1992) were overexpressed in large scale cultures of *E. coli,* strains BL21 (DE3) pLysS, and the protein accumulated in inclusion bodies. Briefly, the transformed BL21 DE3 bacteria were grown in LB medium at 37^{o}C until the cultures reached an OD of 0.6 absorbance units at 600 nm. At this point, isopropyl β-$_{D}$-thiogalactoside (IPTG) was added to a 0.4 mM final concentration and the cultures were incubated for additional 4h at 37^{o}C.

The inclusion bodies were extracted by various rounds of DNAse treatment followed by detergent washes. Specifically: bacteria were centrifuged at 4000rpm for 15 min at 4^{o}C, resuspended in TES (10 mM Tris pH 8, 1 mM EDTA, 100 mM NaCl) buffer and centrifuged again at 4000rpm for 15 min at 4^{o}C. The pellet was resuspended in 10 ml sucrose solution (25% sucrose, 50 mM Tris pH 8, 1 Mm EDTA, 0.1% azide) supplemented with protease inhibitors (1 mM Leupeptin, 1 mM Pepstatin A) and homogenized very well. Lysozyme (20 mg/L of culture, Sigma Aldrich), was added to the sucrose/bacterial lysate mixture, to degrade the bacterial cell wall, and this mix was incubated on ice until viscous (approximately 30 min). After this step, 4 M $MgCl_2$, DNase and RNase (8 mM), EDTA (1 mM) was added followed by homogenization and incubation at room temperature until the DNA had been digested, as judged by decreased viscosity. The mixture was centrifuged at 14000 rpm for 40 min at 4^{o}C and the pellet was resuspended in a Triton X-100/DOC solution [0.5% Triton X-100 (Sigma Aldrich), 0.5% DOC (Deoxycholate), 50 mM Tris pH8, 100 mM NaCl, 0.1% Azide) and 1 mM DTT] and centrifuged at 4000 rpm for 40 min at 4^{o}C. The detergent washes were repeated, with further rounds of DNase/RNase treatment until the inclusion bodies were devoid of debris. Finally, after centrifugation, the pellet was washed by resuspension in Tris buffer (50 mM Tris pH 8, 100 mM NaCl, 0.1% azide) and 1 mM DTT, and centrifugation, for 40 min at 4^{o}C, to remove detergent. Aggregated protein purified from the inclusion bodies was solubilized in 8 M urea/100 mM Tris pH 8/1 mM DTT and spunin an ultracentrifuge at 40 000 rpm for 20 min. The supernatant was collected and the concentration of the protein was quantified using Bradford assay (ThermoScientific). The purity of the inclusion body preparation was assessed by SDS-PAGE and staining with Coomassie Brilliant Blue G-250.

Class I MHC/peptide complexes were refolded by dilution *in vitro* with a synthetic peptide as previously described (Vales-Gomez et al. 1999). Refolded proteins were concentrated using a Kvick Start cassette and purified by size exclusion chromatography using a Sephacryl S-300 column.

Folded MHC proteins eluted within the peak obtained at about 90 ml after sample injection. FPLC fractions containing folded peptide/MHC complexes were pooled, concentrated using a Vivaspin concentrator with 20kDa molecular weight cut-off (Mwco) and exchanged into biotinylation buffer (10 mM Tris-HCl, pH 8) by three cycles of dilution and concentration. Refolded HLA-E heavy chain, β2m and synthetic peptide complexes were biotinylated using d-biotin and BirA enzyme (Source BioScience; GeneCopoeia), exchanged into 20 mM Tris (pH 8.0) and further purified by ion-exchange chromatography (O´Callaghan et al.1998) using a HiTrap Q Sepharose FF column. Correctly biotinylated fractions, identified by SDS-PAGE followed by western blotting with Avidin-HRP, were pooled together and exchanged into PBS buffer by membrane ultrafiltration (mwco: 30k) concentrating the proteins to a volume below 1 ml. For each peptide/MHC complex, the concentration was measured and aliquots were stored at -80^{o}C until use. Biotinylated monomers were assembled into tetramers using standard protocols (http://tetramer.yerkes.emory.edu/support/protocols). Briefly, small aliquots of Extravidin-phycoerythrin (Sigma Aldrich) were added successively to the biotinylated peptide/MHC complex to ensure saturation of avidin-binding sites. The HLA-E tetramer/phycoerythrin complex was then used directly for flow-cytometry staining of whole human blood.

HLA-E and β2m proteins were refolded with the following synthetic peptides: VMAPRTVLL (canonical HLA-B*0702 leader-derived peptide); the *Mycobacterium tuberculosis* derived peptides, p68 (VLRPGGHFL) p62 (RMPPLGHEL) and p55 (VMATRRNVL). The purified synthetic peptides were synthesized by the peptide synthesis service of the CNB proteomics facility.

SDS-PAGE analyses.

SDS-polyacrylamide gel electrophoresis (PAGE) (Electrophoresis running buffer: 195 mM Glycine, 24.8 mM Tris base, 0.1% SDS) analyses were done in reducing or non-reducing conditions. When the samples were analysed under reducing conditions, a 2% SDS loading buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol and bromphenol blue) containing 1 mM DTT was added to the samples and heated at 100^{o}C for 3 min. For analysis under non-reducing conditions a 2% SDS loading buffer with no DTT was used. Proteins were visualized by Coomassie Brilliant Blue stain (0.125% Coomassie blue R-250, 50% Methanol, 10% Acetic acid) by shake for 30´-1h.

For protein transfer onto PVDF (polyvinylidene fluoride) membrane (Immobilon-P.Millipore) transfer buffer (25 mM Tris-base, 192 mM Glycine, 20% methanol) was used. Membranes were blocked with 5% milk in T-TBS (10 mM Trisbase, pH 7.6, 100 mM NaCl, Tween20 0.05%) for o/n. Next day, membranes were washed three times with T-TBS and probed with horseradish peroxidase (HRP)-conjugated Streptavidin (GE Healthcare) in T-TBS for 1h at R.T. Finally, the membranes were washed and visualized using the Amersham ECL western blotting detection kit (GE Healthcare) and X-ray films (Konica Minolta).

Statistical analysis.

Data are represented as the mean values, and error bars show Standard deviations. Statistical analysis was done using GraphPad software (Prism). Multiple data were compared with one-way analysis of variance (ANOVA) or for two variables two-way ANOVA. Studen´s *t*-test was applied to compare pairs of data. p-values < 0.05 were considered statistically significant and are indicated as follows: *p<0.05; **p<0.01; ***p<0.001.

List of reagents:

Reactive	Supplier	Reference
30% Acrylamide/Bis solution	Biorad	161-0156
Anti-PE MicroBeads	MiltenyiBiotec	130-048-801
Biotin-protein Ligase	GeneCopoeia	BIRA500
CFSE	Invitrogen	C34554
DMSO	Sigma	D2650
Ficoll-Paque	GE Healthcare	17-1440-02
Heparin	Sigma	H3149
Human serum	Sigma	H4522
Leupeptin	Calbiochem	108975
Monensin	Sigma	M5273
Paraformaldehyde	Sigma	158127
Pepstatin A	Calbiochem	516481
PVDF transfer membranes	MIllipore	IPVH00010
RandomHexamers	Roche	58002113-01
rh IL-2	Peprotech	200-02
Saponin	Sigma	S7900
SuperScript II RT	Invitrogen	18064-022
T4 DNA ligase	New EnglandBiolabs	M0202S
Tween-20	Sigma	P5927
X-rayfilms	Konica Minolta	

Results

Part I. CD94/NKG2H orphan receptor

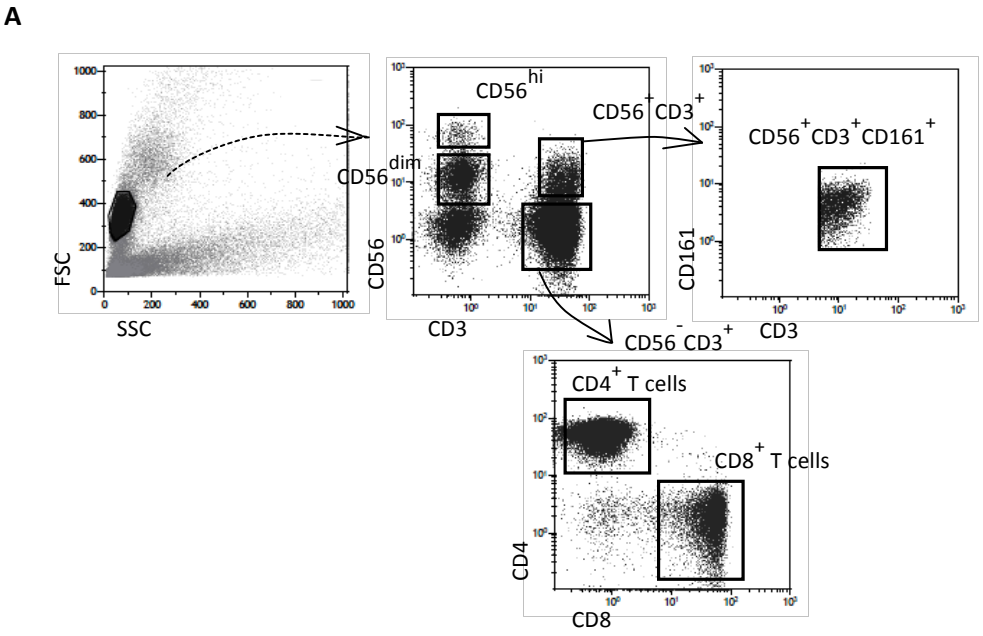
1. NKG2H MOLECULE

During the past 10-12 years the expression and function of the CD94/NKG2A and CD94/NKG2C heterodimeric lectin-like receptors in NK and T cells have been the subject of intensive study (Borrego et al. 2006; Gunturi et al. 2004). While, a series of discoveries have explained several important functions of these receptors as well as of their cognate ligand HLA-E, the first and only report of the existence of the NKG2H protein comes from the study of Teresa Bellón and her coworkers in 1999. These workers studied a αβ $CD8^+$ T cell clone (TCC, K14B06) that could be activated using either mAbs specific for CD3 or CD94. Cell surface labelling and immunoprecipitation experiments identified a protein with molecular weight of 39-kDa that formed a disulphide-linked heterodimer with CD94, but was neither NKG2A nor NKG2C. PCR and cloning identified a novel cDNA clone, a splice variant of NKG2E that these workers named NKG2H. This cDNA was predicted to associate with CD94 and the adaptor molecule DAP12 to form an activating heterodimer.

Further progress in understanding the biology and function of the NKG2H receptor (signalling, molecular interactions and ligand binding) has been seriously hampered by the lack of monoclonal antibodies specific for the NKG2H molecule. When an NKG2H specific mAb (MAB6549, R&D Systems) became commercially available, this gave us the opportunity to study the NKG2H receptor in more detail.

1.1 Phenotypic characterization of $NKG2H^+$ cells in healthy donors

Flow cytometry analysis of freshly isolated PBMCs from 5 healthy individuals was used to identify the cell population expressing NKG2H at the cell surface. A six-colour staining strategy, using a combination of anti-CD3, CD4, CD8, CD56, CD161 and anti-NKG2H mAbs was applied to characterize NKG2H expression on the different PBMC subsets defined by these surface markers. To detect T and NK cells, a lymphocyte gate was first determined by forward and side scatter characteristics (Fig 1-A). T cells were detected by gating the lymphocytes on $CD3^+CD56^-$ cells, while NK cells were defined as $CD3^-CD56^+$ cells. The subpopulation of NK cells, which express higher levels of CD56, was gated as $CD56^{bright}$ and the subpopulation of NK cells expressing lower levels of this marker was gated as $CD56^{dim}$. The subpopulations of T cells were defined as $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells. Additionally, cells that co-expressed the CD56 and CD3 antigens (Mehta et al. 1995) as well as the $CD3^+CD161^+$ (NKT cells) subpopulation were also included in the gating strategy. All these populations were analysed for expression of the NKG2H molecule.



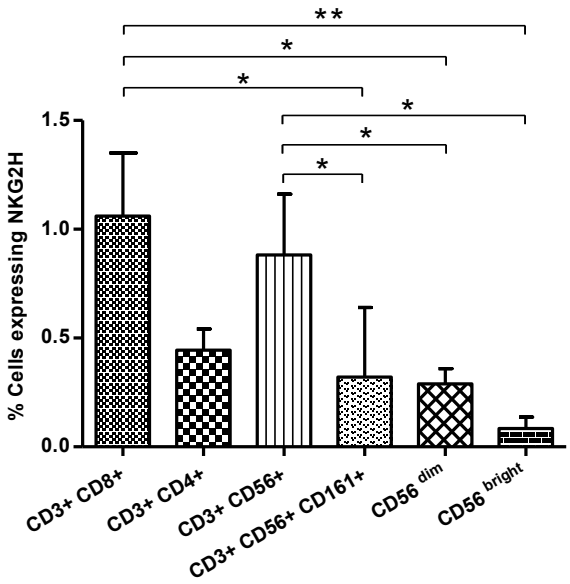


Fig.1: Flow cytometric characterization and quantification of NKG2H expressing cells in PBMCs from healthy individuals. (A) Lymphocyte gate was first determined by forward and sideward scatter parameters. NK cells were defined by gating the lymphocytes as $CD3^{-}CD56^{+}$ cells, which were further divided into $CD56^{bright}$ and $CD56^{dim}$ subsets. $CD3^{+}CD56^{-}$ cells derived from the lymphocyte gate are T cells, where $CD3^{+}CD8^{+}$ and $CD3^{+}CD4^{+}$ are the two cell subsets in this population. Additionally, $CD3^{+}CD56^{+}$ double positive and $CD3^{+}CD56^{+}CD161^{+}$ cells were also gated. NKG2H expression by each gated population was analysed as histogram plots (not shown) **(B)** The percentage of $NKG2H^{+}$ cells detected by gating in the different cell subsets, from 5 different healthy individuals is displayed. Mean data and standard deviation are represented, n=5. Two tailed paired Student test analysis of the logarithm of raw data was used. **p<0.01, *p<0.05.

As shown on Fig. 1-B, NKG2H was expressed on the surface of low, almost undetectable numbers of PBMCs, in the range between 0,5% to 1,5 % of total lymphocytes. Further, substantial donor dependent variations were also observed in the expression of this molecule. The difference of expression of NKG2H receptor between the different cell subsets was small in magnitude but consistently detected in all donors: little or no NKG2H receptor was expressed by NK cells, either the $CD56^{dim}$ or $CD56^{bright}$ subsets; or by $CD3^{+}CD4^{+}$ T cells. On the other hand, the percentage of cells expressing NKG2H was significantly higher for $CD3^{+}CD56^{+}$ and $CD3^{+}CD8^{+}$ cells, suggesting that the regulatory events controlling the expression of this C-type lectin receptor probably act differently on NK and T cells. In the light of these data it is interesting to note that NKG2H was first identified and cloned from a human $CD8^{+}$ T cell clone (Bellón et al. 1999).

2. CD94/NKG2H RECEPTOR AND VIRAL INFECTIONS

The participation of both T and NK cells is necessary for effective defence against HCMV (Mocarski et al. 2001; Pass et al. 2001; Fishman et al. 2007; Dowd et al. 2009; Pawelec et al. 2011; Romo et al. 2011[1]). Patients with congenital HCMV infection have been shown to make $CD8^+$ T cell responses (Marchant et al. 2003; Gibson et al. 2004) and expansion and differentiation of a specific $TCR\gamma\delta^+$ cell subset has also been reported (Vermijlen et al. 2010; Revilleza et al. 2011). The role of NK cells in controlling cytomegalovirus infection is clear in murine systems (Vidal & Lanier. 2006; Mitrović et al. 2012; Miletić et al. 2013) while for humans, a patient deficient for NK cells suffered recurrent herpesvirus infections (Biron, NEJM 1988). The indirect evidence suggesting a role for NK cells in immune responses to HCMV comes from two sources; firstly, the observation that HCMV has evolved/acquired multiple mechanisms of immune evasion that target ligands for NK cell receptors (Wilkinson et al. 2008; Fielding et al. 2014) suggests that immune responses by NK cells have significantly influenced the evolution of the virus. Secondly, study of the evolution of an episode of HCMV infection in a child with a $T^-B^+NK^+$ immunodeficiency has suggested that human NK cells could control CMV infection in the absence of T cells, although the role of virus-specific antibodies was not examined in this case (Kuijpers et al. 2008).

Recently it has been postulated that the CD94/NKG2 lectin-like receptors in the NK cell response to HCMV play important roles in immunity HCMV. As described in the introduction, both CD94/NKG2A inhibitory and CD94/NKG2C activating receptors specifically recognize but differ in their binding affinity for HLA-E that can bind peptide from the leader sequence of the UL40 molecule, stabilizing HLA-E surface expression in HCMV-infected fibroblasts, protecting them against $NKG2A^+$ NK cells (Tomasec et al. 2000; Ulbrecht et al. 2000; Wang et al. 2002; Heatley al. 2013).

Several NK cell subsets, according to the expression of CD94/NKG2 receptors have been characterized in human peripheral blood: $NKG2A^+/C^-$, $NKG2A^-/C^+$, $NKG2A^+/C^+$, and $NKG2A^-/C^-$. In HCMV seronegative individuals, $NKG2A^+/C^-$ and $NKG2A^-/C^-$ NK cells are predominant whether the other cell subsets are negligible. However, an $NKG2C^{bright}NKG2A^-NKp30^{low}NKp46^{low}$ cell population has been shown to expand in HCMV seropositive individuals (Muntasell et al. 2013[1]). An increased proportion of $NKG2C^+$ NK and T cells have also been reported in healthy HCMV seropositive children (Noyola et al. 2012). These $NKG2C^{bright}$ cells have been shown to be functionally mature, mediating cytotoxicity and cytokine production, express perforin and granzyme B and predominantly display inhibitory KIR for self HLA-C molecules (Bjorkstrom et al. 2011; Bezait et al. 2013; Djaoud et al. 2013; Muntasell et al. 2013[2]). The magnitude of the $NKG2C^+$ NK cell expansion was shown to be quite variable, being undetectable in some $HCMV^+$ individuals whereas in others it could represent >50% of the NK-cell compartment. These $NKG2C^+$ NK cells were shown to persist and be stable under steady state conditions during the lifetime of the individual, leading to the idea that expression of NKG2C marks the ¨memory-like¨ cells of the NK cell population (Min-Oo et al. 2013; Muntasell et al. 2013[1]).

Alignment of the NKG2H peptide with other members of the NKG2 C-type lectin like family (Fig.3 Introduction part) shows that NKG2E/H and the NKG2C family members are highly similar in the amino acid sequence of the cytoplasmic and transmembrane domain but more divergent in their extracellular ligand-binding region. Interestingly, all three members share the same charged lysine residue in the transmembrane domain responsible for the DAP12 adaptor protein association. As mentioned previously, the functional properties of NKG2H are still unknown but given that $NKG2C^+$ cells expand in HCMV seropositive individuals and have a possible role in the control of the HCMV infections, the structural similarity between NKG2H and NKG2C molecules, made it seem reasonable to ask how viral infections, and in particular HCMV infections, affected $NKG2H^+$ cells. To address this issue, we analysed the expression of CD94/NKG2A, NKG2C and NKG2H receptors in peripheral blood mononuclear cells (PBMCs) co-cultured with HCMV infected fibroblasts.

2.1 Expansion of CD94/$NKG2H^+$ cells in response to HCMV infected fibroblasts.

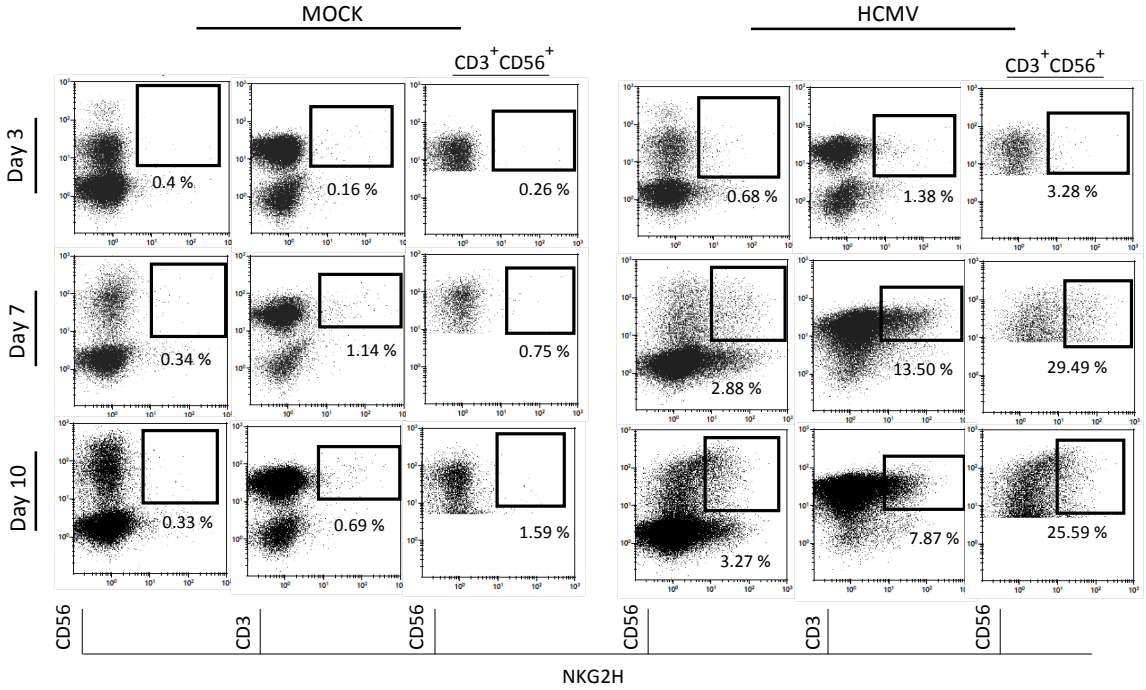
An *in vitro* culture system for stimulation of PBMCs with HCMV infected fibroblasts has been widely used to study cytotoxic T lymphocytes (CTLs) specific for viral antigens and described elsewhere (Reusser et al. 1991). We used this system to address our first objective, to establish the effect of culture with HCMV infected fibroblasts on NKG2H expression in lymphocytes. PBMCs purified from healthy donors were co-cultured either with mock or HCMV infected fibroblasts, as described by Guma et al. 2006 with the exception that infections were done with HCMV strain Merlin, which is a low passage clinical strain. The phenotype of the PBMCs, in particular NKG2A, -C and –H expression, were compared, using flow cytometry, at different time points: day 3, day 7 and day 10.

Three-color immunofluorescence analyses of the PBMCs at the end of each co-culture period revelled a marked increase in the proportion of $NKG2H^+$ cells in PBMCs co-cultured with HCMV infected fibroblasts compared to the PBMCs co-cultured with mock infected fibroblasts (Fig. 2). This increase in the percentage of $NKG2H^+$ cells was detected first at day 3 and further expansion of these cells was observed by day 7 and day 10 (Fig.2-A).

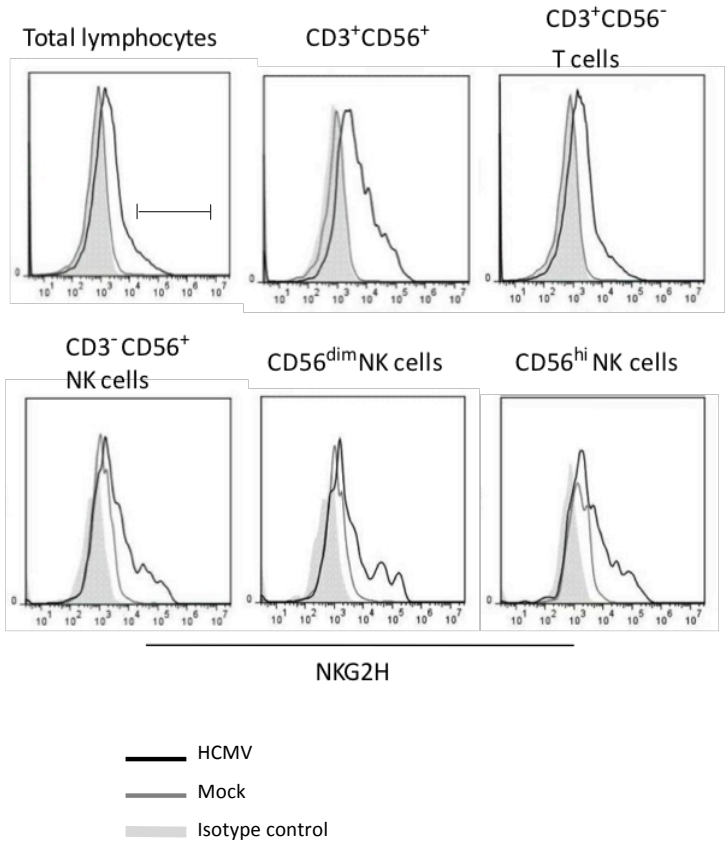
The change of the expression of NKG2H receptor in the different populations of cytotoxic lymphocytes was analysed using anti-CD3 and anti-CD56 mAb´s to define NK and T cell populations (Fig. 2-B and Fig. 2-C). Cell surface expression of NKG2H was clearly increased on lymphocytes that had encountered HCMV infected cells comparing to the mock infected controls. After 7 days, as well as after 10 days of cell culture, a significant increase of the percentage of NKG2H expressing T cells, NK cells, both $CD56^{dim}$ and $CD56^{hi}$ subsets, as well as $CD3^+CD56^+$ cells was observed.

Closer analyses at the different time points of co-culture between the PBMCs and the mock or HCMV infected fibroblasts indicated that the proportion of $NKG2H^+$ T cells ($CD3^+CD56^-$) increased most during the first week, but by day 10 the numbers of these cells had started to decrease. In contrast, the percentage of $NKG2H^+$ cells within the NK cell population ($CD3^-CD56^+$) continued to increase from 35% at day 7 to 40% at day 10 (Fig. 2-C).

A


MOCK
HCMV
CD3+CD56+
CD3+CD56+
Day 3
Day 7
Day 10
0.4 %
0.16 %
0.26 %
0.68 %
1.38 %
3.28 %
0.34 %
1.14 %
0.75 %
2.88 %
13.50 %
29.49 %
0.33 %
0.69 %
1.59 %
3.27 %
7.87 %
25.59 %
CD56
CD3
CD56
CD56
CD3
CD56
NKG2H

B


Total lymphocytes
CD3+CD56+
CD3+CD56-
T cells
CD3-CD56+
NK cells
CD56dim NK cells
CD56hi NK cells
NKG2H
HCMV
Mock
Isotype control

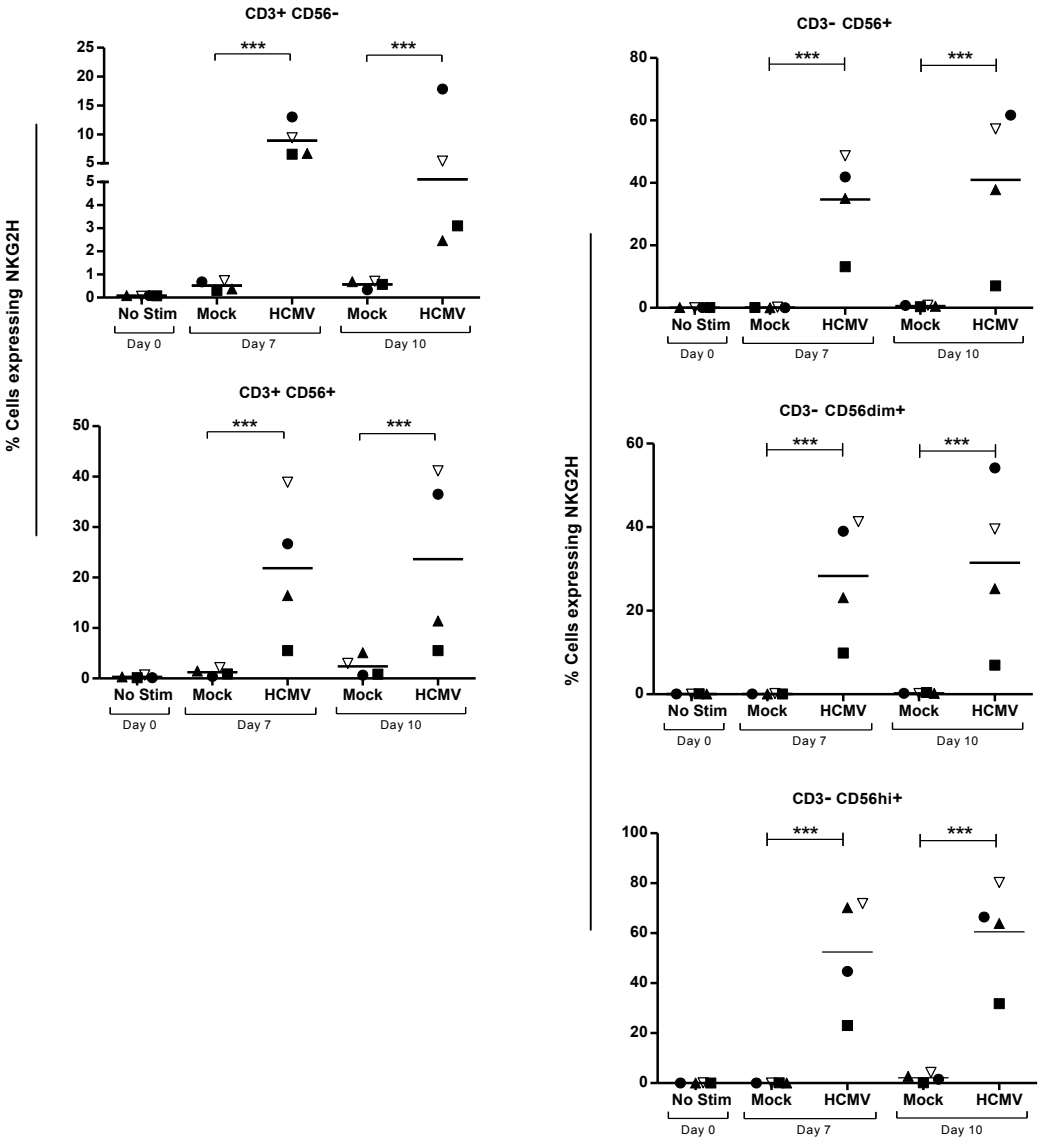


Fig.2: An NKG2H$^+$ cell expansion was induced in vitro by co-culture of lymphocytes with HCMV infected fibroblast. (A) Fibroblasts were mock or HCMV (Merlin) infected overnight, and then co-cultured with PBMCs. These co-cultures were maintained for 3, 7 and 10 days, then harvested and stained for surface markers as described in ¨Materials and methods¨. A representative result of one healthy donor is shown for the expression of NKG2H in CD56$^+$ cells (first and fourth panels), for the expression of NKG2H in CD3$^+$ cells (second and fifth panels) and for the expression of NKG2H in CD3$^+$CD56$^+$ cells (third and sixth panels). **(B)** Change of the cell surface expression of NKG2H by the different lymphocyte subsets after co-culture with mock or HCMV (Merlin) infected fibroblasts. Representative histograms of NKG2H expression on the different lymphocyte subsets are shown; grey filled – Isotype control, grey line – mock infected fibroblasts, black line – HCMV (Merlin) infected fibroblasts. **(C)** Analyses of the percentage of NKG2H$^+$ cells expressed by CD3$^+$CD56$^-$, CD3$^+$CD56$^+$, CD3$^-$CD56$^+$, CD3$^-$ CD56^{dim+}, CD3-CD56^{hi+} after co-culture with Mock of HCMV infected fibroblasts. Each symbol corresponds to the result obtained from an individual donor; filled symbols – HCMV seronegative, open symbol – HCMV seropositive. Mean data and standard deviations are represented, n=4; ***p<0.001.

2.2 CD94/NKG2H, but not NKG2A or NKG2C positive cells expand in response to HCMV infected fibroblasts.

In parallel to the analysis of NKG2H expression, changes in the proportion of NKG2C and NKG2A molecules on PBMCs stimulated with mock or HCMV (Merlin) infected fibroblasts were also analysed (Fig. 3). As previously seen, a 10-fold increase of the percentage of NKG2H$^+$ cells was observed after co-culture of PBMCs with HCMV infected fibroblast compared with the mock controls, but no changes in the frequency of NKG2C or NKG2A positive cells between the two conditions were observed. Given that the majority of our healthy donors were CMV seronegative, these observations agree with the published data of Guma et al. 2006 which describe comparable levels of NKG2A receptor expression on PBMCs after culture with mock or HCMV infected fibroblast whereas expansion of NKG2C$^+$ cells was observed in PBMCs from 6/11 HCMV seropositive donors cultured with virus infected fibroblasts, but never in PBMCs from seronegative donors.

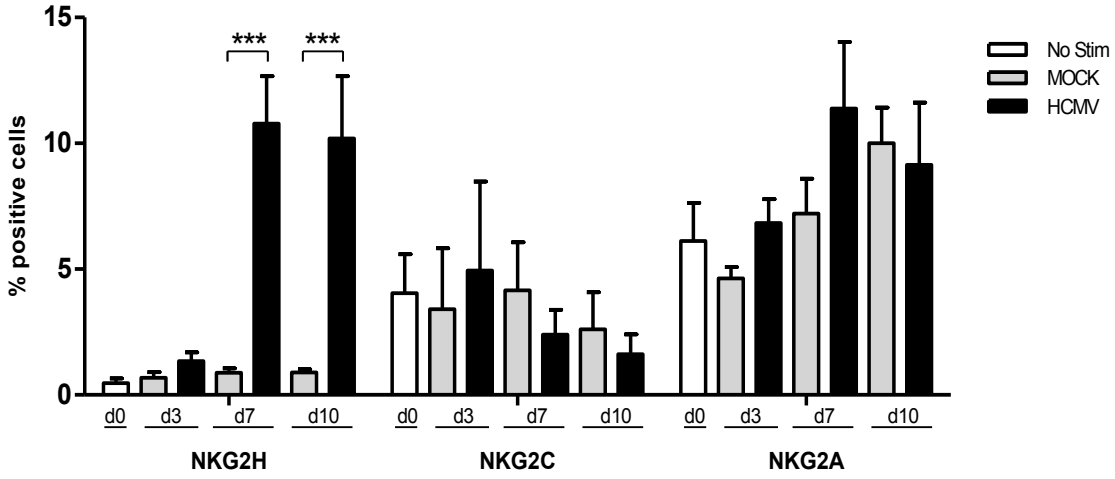


Fig. 3: NKG2 receptor expression in PBMCs after stimulation with HCMV infected fibroblasts. PBMCs from healthy individuals were co-cultured with either mock or HCMV (Merlin) infected fibroblasts for 3, 7 and 10 days (50 U/ml of IL2 was added after day 3). The percentage of cells expressing the different NKG2 receptors was assessed at the different time points. Day 0 is the point before culturing the cells *in vitro*. Mean data and standard deviations are represented, n=8. The proportion of NKG2H$^+$, NKG2A$^+$ or NKG2C$^+$ cells detected in the PBMCs from 8 different donors, in three separate experiments are shown; ***p<0.001.

Given that there was a clear expansion of NKG2H expressing cells after the 7 days of PBMCs infected fibroblast co-culture, the next sets of experiments were analysed after 7 days of culture in this system.

In parallel, NKG2H mRNA expression by lymphocytes stimulated with mock or HCMV infected fibroblasts was evaluated in samples of total RNA, isolated from these cells at different time points. Surprisingly, no significant differences were observed in NKG2H mRNA expression, suggesting that NKG2H surface expression must be regulated post-transcriptionally and/or post-translationally rather than at the level of transcription (Fig. 4).

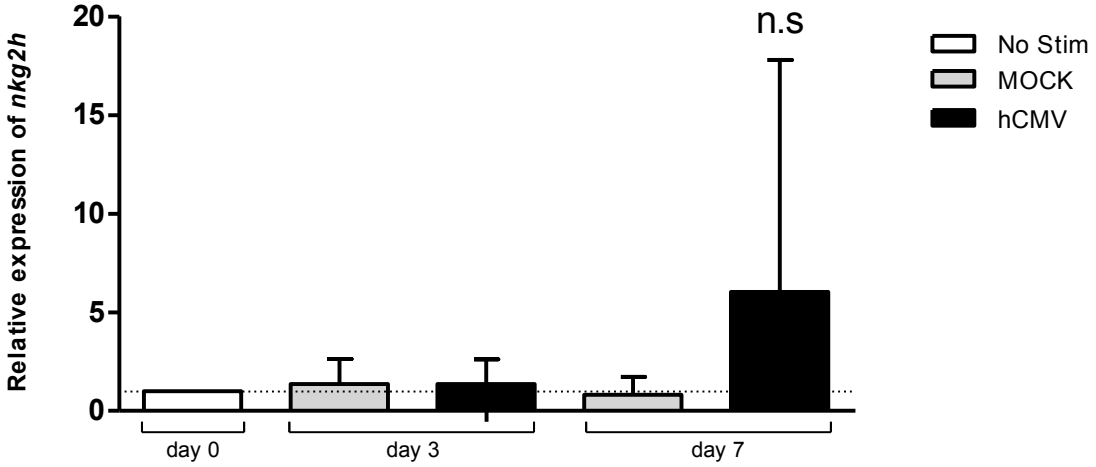


Fig. 4: Relative expression of the *nkg2h* gene measured by quantitative PCR. Total mRNA was extracted from PBMCs stimulated with mock or HCMV (Merlin) infected fibroblasts for 3 or 7 days as described in ¨Materials and Methods¨. The amount of NKG2H mRNA expression was normalized against the quantity of β-actin mRNA expression in each sample, and related to the NKG2H expression in control, no stimulated cells (dotted line). Mean data and standard deviations are represented, n=4 in one experiment. No significant differences were found.

2.3 Contact between PBMC and HCMV infected fibroblasts is required for efficient expansion of CD94/NKG2H$^+$ cells.

In order to define the relative importance of soluble factors and cell:cell contact for the expansion of the NKG2H$^+$ cells, PBMCs were cultured with HCMV (Merlin) or mock infected fibroblasts either in the same chamber or in different chambers of a transwell separated by a cell-impermeable membrane that permits diffusion only of soluble factors. Under these conditions a small increase in the percentage of NKG2H$^+$ cells was observed when the PBMC were separated from the infected cells whereas marked induction of NKG2H expression was observed only when the PBMC were cultured in the same chamber as the virus-infected fibroblasts (Fig. 5). These data indicate that direct interaction between PBMCs and HCMV infected fibroblasts is required for optimal induction of NKG2H$^+$ cell expression.

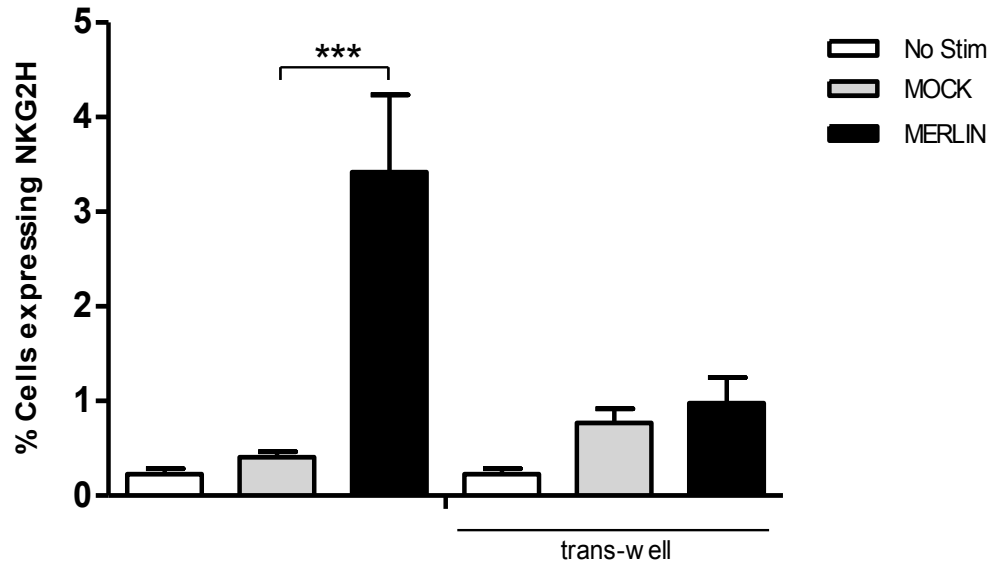


Fig. 5: Direct contact with the HCMV (Merlin) infected fibroblasts is required for the expansion of NKG2H⁺ cells. Purified PBMCs were cultured with mock or HCMV (Merlin) infected fibroblasts in same chamber, or in different chambers separated by a semi-permeable membrane for 7 days before cell surface staining. Mean data and standard deviations are represented, n=6. The proportion of NKG2H⁺ cells in PBMCs from 6 different donors, in two separate experiments are shown;***p<0.001.

2.4 NKG2H⁺ cell subset expansion in response to different HCMV strains and other viruses.

HCMV strains display different levels of virulence, tissue tropism and pathogenicity depending on their degree of adaptation in fibroblasts. HCMV strain Merlin is a minimally passaged clinical strain, passaged only twice *in vitro* before the genome was cloned in a bacterial artificial chromosome for propagation. The HCMV strains Toledo and TB/40 are widely used laboratory models of clinical strains whereas AD169 is a highly passaged laboratory HCMV strain. Clinical HCMV strains are more virulent (Elek et al. 1974; Quinnan et al. 1984; Plotkin et al. 1976) and also confer a stronger resistance to NK cell attack than the highly-passaged attenuated strains which produce only marginal effects with respect to NK cell recognition (Cerboni et al. 2000) suggesting that at least some of the mechanisms employed to evade NK cell lysis may be lost during *in vitro* passage of this viruses.

To establish whether other HCMV strains than the viral strain Merlin could provoke the same NKG2H expansion effect on PBMCs, we compared the effects on PBMC of co-culture with fibroblasts infected with strains AD169, Toledo and TB/40 and the previously used strain Merlin. NKG2H expression was checked at the day 3 and day 7 time points (Fig. 6). Under these conditions only PBMCs co-cultured with fibroblasts infected with the viral strains Merlin and TB/40 were able to drive the expansion of NKG2H$^+$ cells. In contrast, co-culture of PBMCs with fibroblasts infected with the clinical strain Toledo or the highly passaged strain AD169 did not lead to the appearance of an NKG2H expressing cell population.

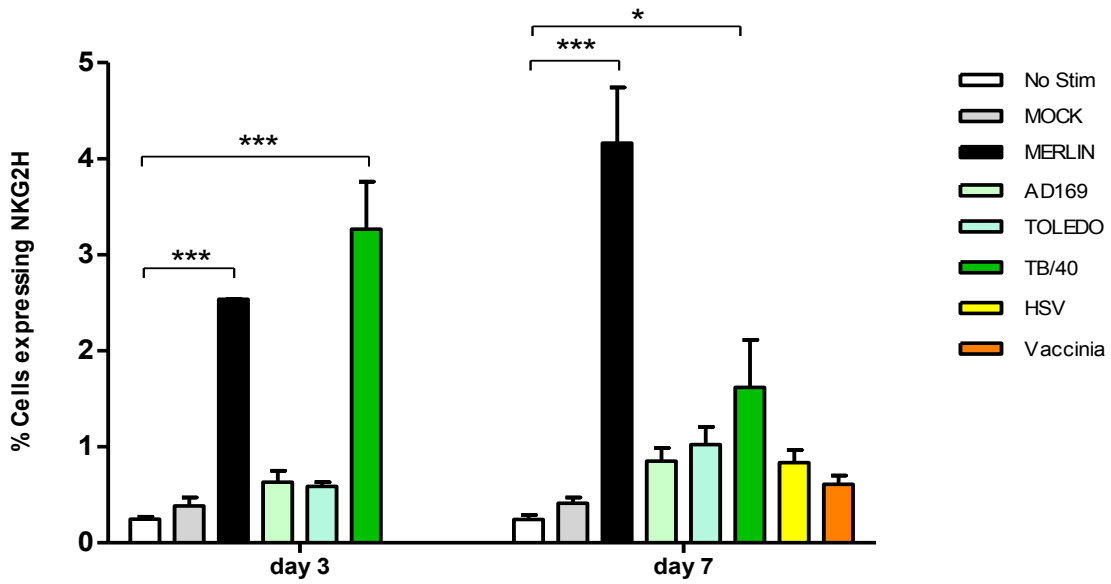


Fig. 6: HCMV strains Merlin and TB/40 but not the viral strains Toledo and AD169 infected fibroblasts are capable to drive expansion of NKG2H⁺ cells. hTERT fibroblasts were infected with different type of viruses as well as different genetic variants of the same HCMV as indicated in the legend of this figure. The percentage of NKG2H⁺ cells was examined at day 0 (before cell stimulation), day 3 and day 7. For HSV and Vaccinia the percentage of NKG2H⁺ cells was assessed only at day 7. Mean data and standard deviations are represented, n=8. The proportion of NKG2H⁺ cells in PBMCs from 8 different donors, in three separate experiments are shown; *p<0.05, ***p<0.001.

Similarly, no significant increase of the percentage of NKG2H$^+$ cells was noted upon incubation of PBMCs with mock, herpes simplex virus (HSV) or Vaccinia virus infected fibroblasts. Together, these data indicate that the changes in NKG2H expression associated with virus infection depend not only on the type of the virus but also on the genetic variant or subtype of the studied virus.

2.5 Loss of NKG2H expression after removal of PBMCs from contact with infected fibroblasts.

As described above in the introduction to this results section, expansion of NKG2C$^+$ cells has been associated with prior HCMV infection. These cells have been described to be more potent producers of IFNγ and to express CD57, a marker of terminal differentiation, suggesting the existence of a secondary response against CMV antigen (Foley et al. 2012). Moreover, the expansion of memory-like NKG2C$^+$ NK cells was found to be persistent, although the basis of this persistence is not clear (López-Botet et al. 2014).

As a first approximation to check whether cells expressing the CD94/NKG2H receptor could also be classified as showing this memory like phenotype PBMCs were co-cultured with mock or HCMV (Merlin) infected fibroblasts for 7 days. After this time, the percentage of NKG2H$^+$ cells was assessed and cells were left to rest in 50 U/ml of IL2, for four to five days in culture. As usual, a clear expansion of NKG2H$^+$ cells was observed after 7 days of the co-stimulation period

(Fig. 7). In contrast, once the cells were separated from the infected fibroblasts and rested for four to five days with IL2, they lost the surface expression of NKG2H. These preliminary data indicate that the expanded NKG2H expressing cells are not persistent over time and this molecule cannot be considered as a memory-like marker for HCMV infection, at least in this model system.

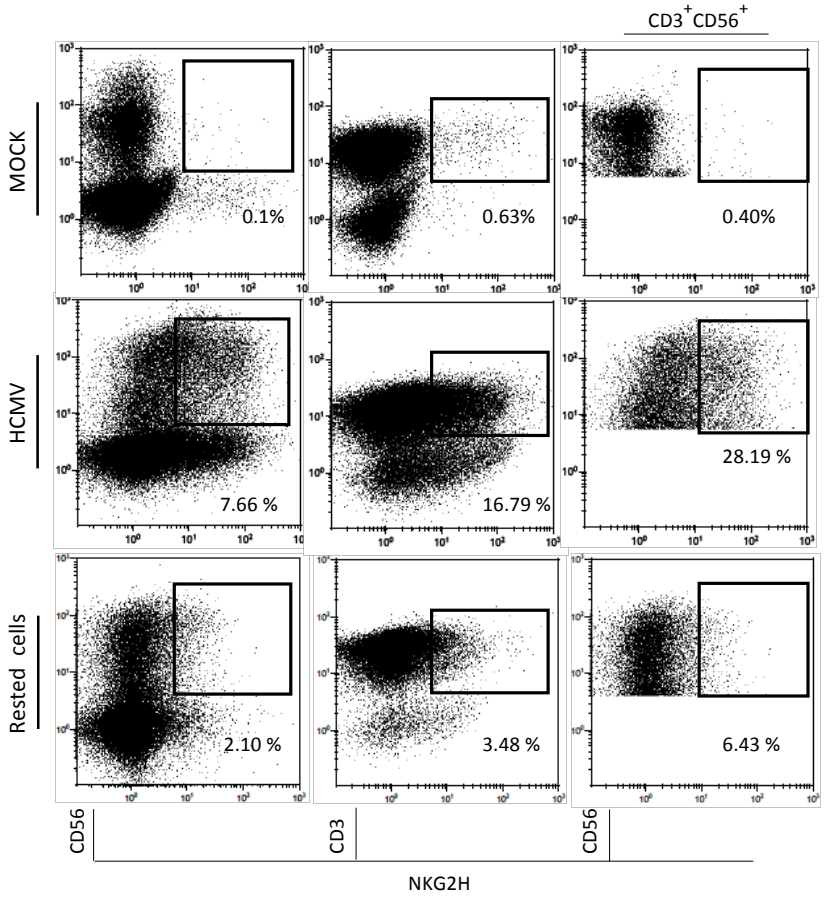
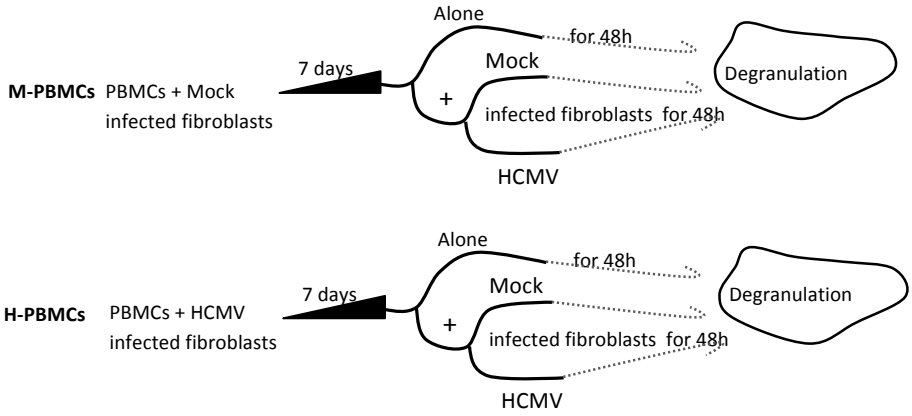


Fig. 7: Expanded NKG2H⁺ cells go back to normal levels when PBMCs are not in contact with the infected fibroblasts. After 7 days of co-culture between PBMCs and mock or HCMV infected fibroblasts, aliquots of the cells were collected and stained for surface CD3, CD56 and NKG2H molecule expression while the others were left to rest for four-five days with 50 U/ml of IL2. After this period of time, cells were stained with anti CD3, CD56 and NKG2H antibodies. Representative flow cytometry dot plots show the percentage of gate $CD56^{+}NKG2H^{+}$, $CD3^{+}NKG2H^{+}$ and $CD3^{+}CD56^{+}NKG2H^{+}$ cells.

2.6 Degranulation capacity of PBMC in response to HCMV infected fibroblasts

Other data indicated that mAb-mediated ligation of the NKG2H receptor led to increased T cell death in systems involving polyclonal T cell activation (sections 3.1 and 3. 4). One testable prediction from these data is that induction of NKG2H expression (and the consequent death of activated lymphocytes) could be associated with weaker secondary immune responses. In order to test this idea the functional capacity of (PBMCs) NK and T cells was assessed in the model

system involving the culture of PBMC with mock or HCMV infected fibroblasts. As NKG2H receptor expression was seen to increase transiently at the cell surface in response to HCMV infected fibroblasts, PBMCs were co-cultured with mock or HCMV infected fibroblasts for 7 days and then re-stimulated by co-incubation with new cultures of either mock or HCMV infected fibroblasts for another 48h (Scheme 1 and Fig. 8). Lymphocyte activation was assessed by examining the expression of CD107a, a degranulation marker, on the lymphocytes in the culture after the second incubation. Allogeneic stimulation (HLA-type: HLA-A*01, B*08/*41, C*07/*17, DRB1*03/*07. $DRB3^+$, $DRB4^+$), induced by the HLA antigens expressed on the fibroblasts led to increased CD107a expression by T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$) and effector cells ($CD3^+CD56^+$) present in the PBMCs when cultured with mock infected fibroblasts (M-PBMCs). In contrast, the same T cells, NK cells and double positive cells degranulated less on exposure to HCMV-infected fibroblasts (H-PBMCs) (Fig. 8). This presumably reflects the downregulation of HLA class I molecules and ligands for activating NK receptors that is known to occur after HCMV infection (Wilkinson et al.2008; Jackson et al. 2011). Secondary exposure of M-PBMCs (PBMCs previously co-cultured with mock infected fibroblasts for 7 days) to mock infected allogeneic fibroblast (MOCK inf. fibroblast + M-PBMCs) was associated with an increase in the percentage of degranulating T and NK cells but a decrease in the percentage of degranulating $CD3^+CD56^+$ cells. A round of culture of these same M-PBMCs with HCMV-infected fibroblasts (HCMV inf. fibroblasts + M-PBMCs) was associated with increased degranulation of the NK cells and double positive cells but with no change in degranulation of the T cells compared to the degranulation of M-PBMCs alone. Secondary exposure of H-PBMCs (PBMCs cultured with HCMV infected fibroblasts for 7 days) to either mock (MOCK inf. fibroblasts + H-PBMCs) or HCMV infected fibroblasts (HCMV inf. fibroblasts + H-PBMCs) was not associated with any significant change in degranulation of the NK cells, $CD3^+CD56^+$ cells and T cells compared to the degranulation of H-PBMCs alone (Fig. 8). However a major limitation of these experiments is that it is difficult to ascribe the effects observed to the action of NKG2H. The use in these experiments of $F(ab)_2$ fragments of CD94 specific mAbs would give some information, but since at least some of the cells under study may also express CD94/NKG2A and CD94/NKG2C these experiments could not be interpreted definitively and at present the preparation of $F(ab)_2$ fragments of the commercially available NKG2H mAb is not feasible.



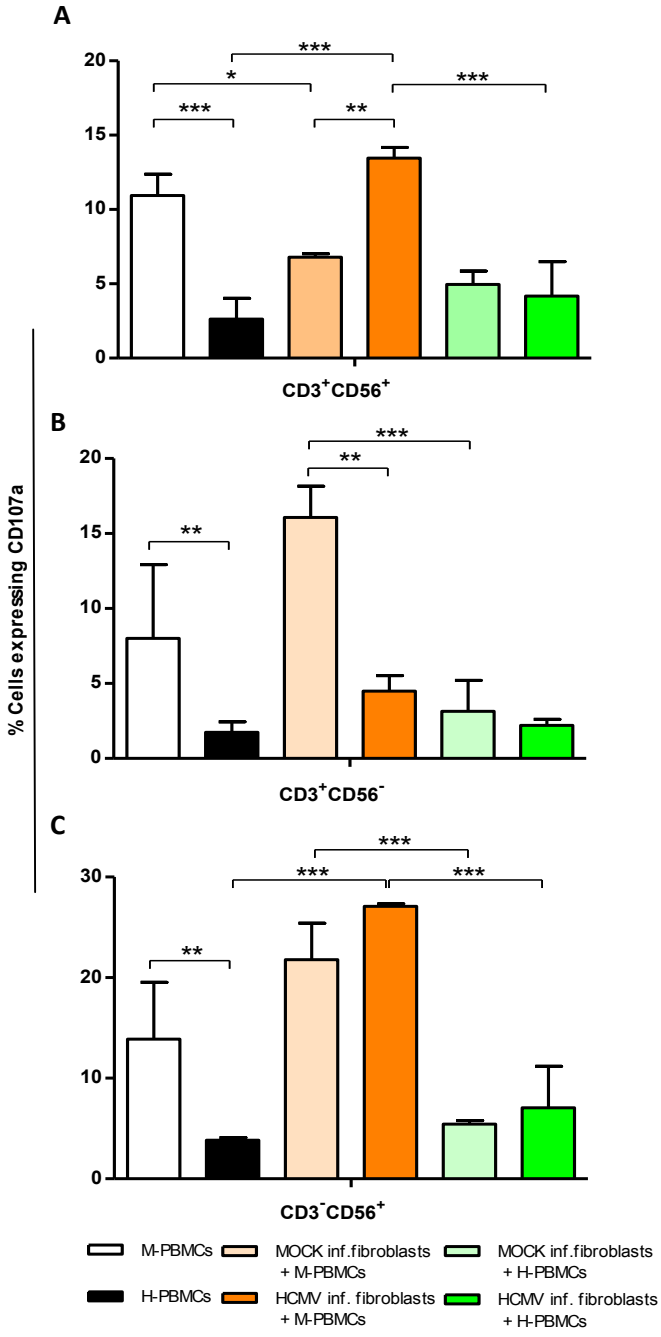


Fig. 8: Degranulation of PBMCs to mock or HCMV infected allogeneic fibroblasts. PBMCs were co-cultured with mock or HCMV infected fibroblasts for 7 days. Then, cells were collected and re-stimulated with freshly infected mock or HCMV infected fibroblasts for another 48h. Cell surface expression of CD107a was assessed after 5h incubation period with anti-CD107a antibody added to the media. Cells were co-stained for CD3 and CD56 surface markers. Percentage of $CD3^{+}CD56^{+}$ double positive cells (A) $CD3^{+}CD56^{-}$ T cells (B) and $CD3^{-}CD56^{+}$ NK cells (C) expressing CD107a on cell surface is presented. M-PBMCs (PBMCs co-cultured with mock infected fibroblasts for 7 days); H-PBMCs (PBMCs co-cultured with HCMV infected fibroblasts for 7 days); inf. fibroblasts (infected fibroblasts). Mean data and standard deviation are presented, n=2. *p<0.05, **p<0.01, ***p<0.001

3. FUNCTIONAL EFFECTS OF NKG2H RECEPTOR LIGATION BY SPECIFIC ANTIBODY

In the paper by Bellón et al. 1999, NKG2H was described as possible partner forming a heterodimer with CD94 protein that associated with the DAP12 adaptor molecule to transmit activating signals. The engagement of this heterodimer expressed on the K14B06 T cell clone by anti CD94 antibody, triggered both, early activation events such as Ca^{2+} mobilization, and late effector functions such as induction of TCR independent cytolytic activity and IFN-γ production. This observation was surprising since, in general, activating NK receptors expressed by T cells, only act as co-activators molecules, modulating the TCR-mediated response but never inducing cytolytic activity. Moreover, the evidence implicating NKG2H as part of an activating heterodimer on this T cell clone was all indirect, thus the possibility that some other activating receptor could associate with the CD94 heterodimer and be responsible for some of the observed effects could not be formally excluded.

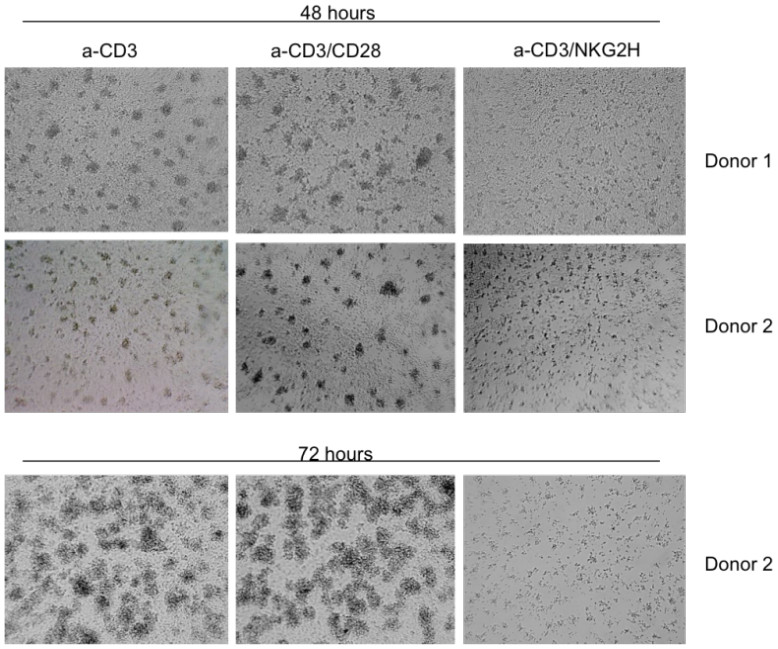
To try and establish the functional consequences of ligation of the NKG2H receptor, purified mAb specific for this molecule was immobilised on plastic and used to stimulate peripheral blood mononuclear cells derived from healthy donors in the presence and absence of suboptimal doses of a stimulatory anti-CD3 mAb.

3.1 Activation of NKG2H expressing cells markedly reduces expression of the activation marker CD69 by the other cells in the culture.

In general, optimal activation of T cells is thought to require at least two types of signals: antigen dependent signals through the T cell receptor - CD3 complex and antigen independent co-stimulation. This combination of stimuli can be modelled *in vitro* using immobilized anti-CD3 alone or in combination with immobilized anti-CD28 antibody to induce T cell activation and proliferation (Geppert et al. 1988; Thompson et al. 1989).

Since the CD94/NKG2H receptor was found to be preferentially expressed on $CD3^+$ T cells (Fig. 1B), PBMCs were stimulated using a suboptimal dosage of immobilized anti-CD3 antibody (1 μg/ml) in combination with anti-NKG2H antibody (5 μg/ml), for 48h or 72h. PBMC stimulation with either immobilized anti-CD3 antibody alone or a combination of anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibody were used as controls. Simple visual inspection using light microscopy revealed that cultures stimulated with anti-CD3/NKG2H antibodies appeared far less activated than the control cells after 48h of stimulation and these differences were much more pronounced after 72h (Fig. 9-A). To confirm that cell cultures stimulated through NKG2H were less activated than the cells receiving anti-CD3 or anti-CD3/CD28 stimulation, the expression of CD69, a marker of early cell activation (Ziegler et al. 1994) was assayed.

A



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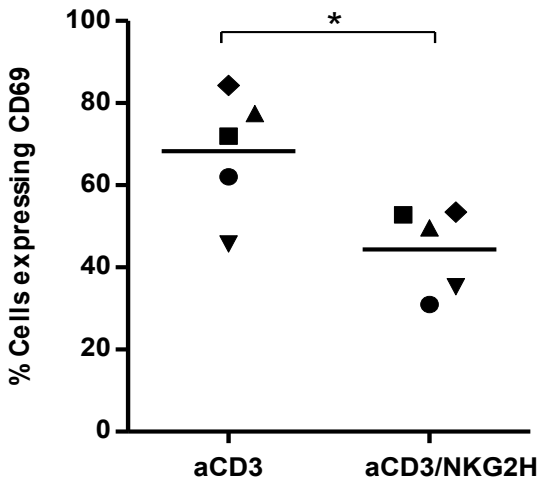


Fig. 9: Stimulation of PBMCs with antibodies directed to CD3 and NKG2H decreases lymphocyte activation. (A) Microscope images of PBMCs from two representative healthy donors incubated in plates pre-coated with anti-CD3 (1 μg/ml), the combination of anti-CD3 and CD28 (1 μg/ml) or anti-CD3 and NKG2H [(1 μg/ml)/ (10 μg/ml)] antibodies for 48h (Donor 1) or 48h and 72h (Donor 2). **(B)** Quantification of the percentage of cells expressing CD69 after anti-CD3 or anti-CD3/NKG2H stimulation. Each symbol corresponds to the result obtained from an individual donor. Mean data are represented, n=5; *p<0.05. a - anti

Analysis of the flow cytometry staining indicated a significant decrease of the percentage of $CD69^{+}$ cells in cultures stimulated with anti-CD3/NKG2H antibodies compared to cells stimulated only with anti-CD3 (Fig. 9-B).

3.2 Activation of NKG2H expressing cells markedly reduces the proliferation of the other cells in the culture.

As NKG2H stimulation was observed to impair T cell activation in the culture, the effects of stimulation through NKG2H on the cell proliferative response were also measured. For these experiments, purified PBMCs were stained with CFSE dye and cultured in plates pre-coated with anti-CD3/NKG2H or anti-CD3 antibodies. The proliferation of the stimulated cells was assessed at different time points: days 2, 3 and 4 (Fig. 10-A and Fig. 10-B).

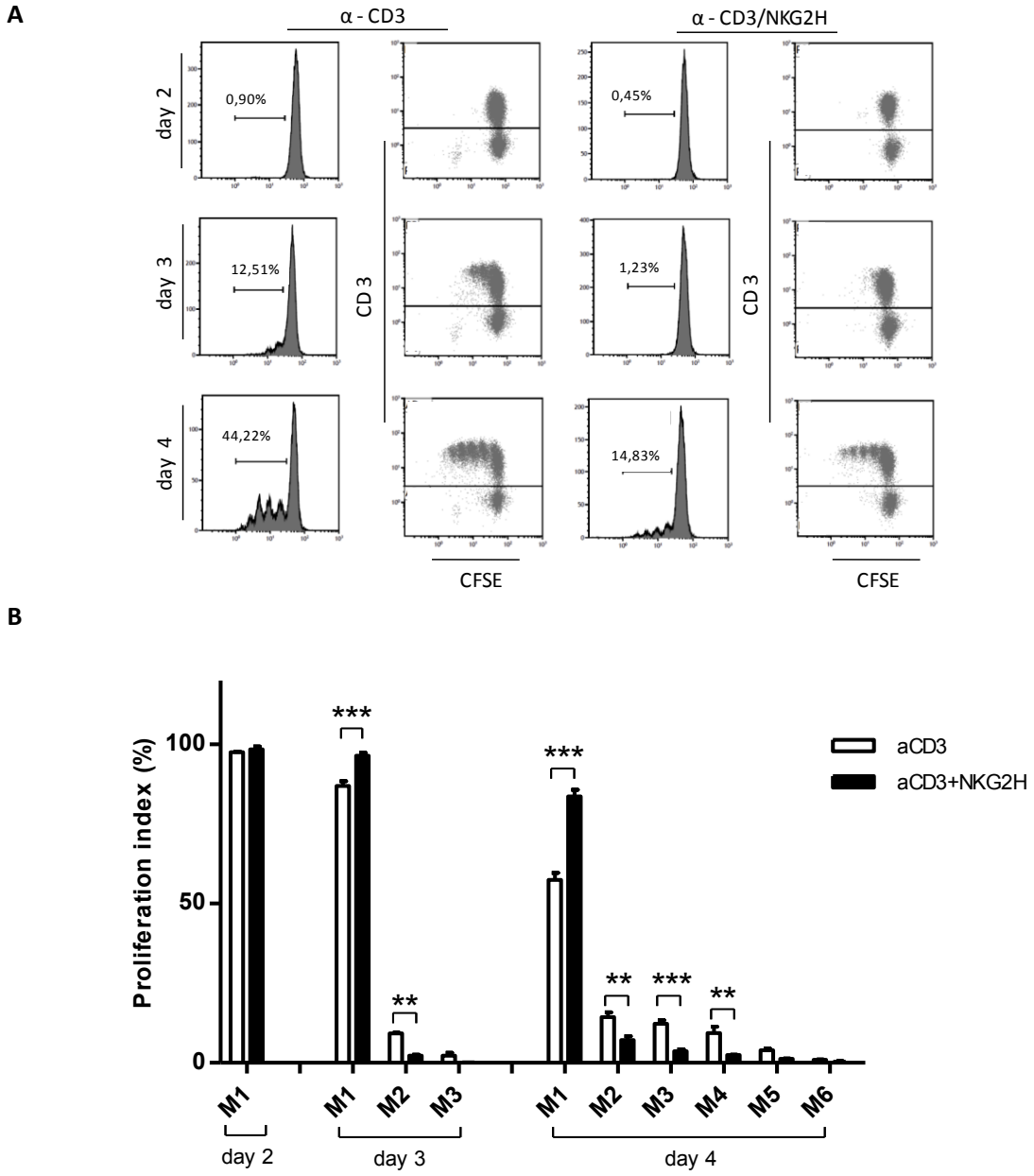


Fig. 10: NKG2H stimulation reduces proliferation of PBMCs in the cell culture. (A) Representative flow cytometry histograms and dot plots of CFSE-labelled PBMCs stimulated with anti-CD3 (1 μg/ml) or anti-CD3 and NKG2H [(1 μg/ml)/ (10 μg/ml) respectively] for 2, 3 or 4 days. Analyses are gated on lymphocytes (based on forward and side scatter). **(B)** Proliferation index was calculated and is represented as a column graph. Mean data and standard deviations are represented, n=2 in two independent experiments; **p<0.01, ***p<0.001. a – anti.

These data showed a significant reduction in proliferation of the cultured cells when stimulated using mAbs to CD3 and NKG2H, compared to stimulation with anti-CD3 only (Fig. 10-B).

3.3 Reduced activation of cell in the culture is characteristic for NKG2H but not for NKG2A or NKG2C receptor signalling.

To test whether the observed reduction in T cell activation after anti-CD3/NKG2H stimulation is a characteristic specific for CD94/NKG2H receptor signalling, these experiments were repeated using PBMCs cultured in plates where combinations of immobilized anti-CD3/NKG2C and anti-CD3/NKG2A had been immobilised. PBMCs were once again purified from healthy individuals and plated in the presence of anti-CD3 antibody or anti-CD3 combined with specific mAb for each of the NKG2A, -C and -H receptors, used at the same concentration. The percentage of cells induced to express the activation marker CD69, was significantly decreased in the cell cultures where PBMCs were co-stimulated with anti-CD3 and anti-NKG2H antibody. In contrast, the phenotype of the PBMCs stimulated by anti-CD3/NKG2C or anti-CD3/NKG2A was comparable to the control anti-CD3 stimulation (Fig. 11).

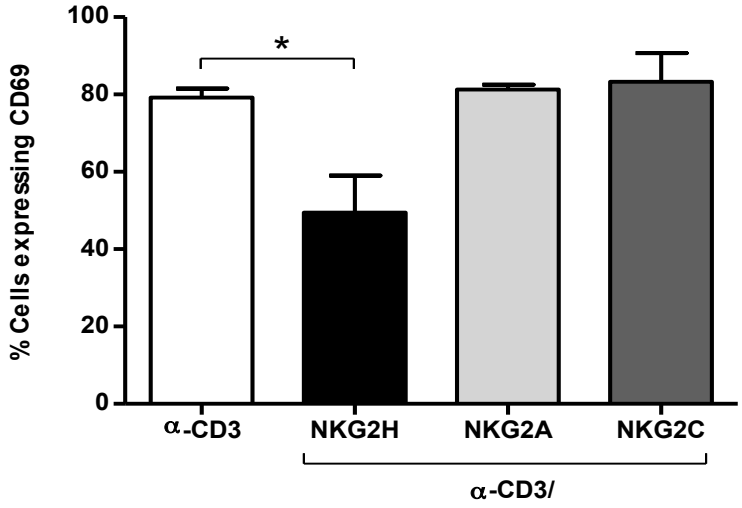


Fig. 11: NKG2H but not NKG2A or NKG2C signaling leads to reduced PBMCs activation in the cell culture.24 well plate was covered with anti-CD3 (1 μg/ml), anti-CD3/NKG2A [(1 μg/ml)/ (10 μg/ml)], anti-CD3/NKG2C [(1 μg/ml)/ (10 μg/ml)] or anti-CD3/NKG2H [(1 μg/ml)/ (10 μg/ml)] antibody. Purified PBMCs from healthy donors were isolated, and cultured in the antibody-coated plates for 48h. After this time, the cells were collected and stained with anti-CD69 antibody. Mean data and standard deviations are represented, n=2 ; *$p<0.05$. α– anti.

3.4 Activation of NKG2H$^+$, but not NKG2A$^+$ or NKG2C$^+$ cells induces apoptosis of other cells in the culture.

The previous observations were surprising since they indicated that activation of the 0.5 - 1.5% of T cells expressing NKG2H could modulate the activation of the remaining 99.5 – 98.5% of T cells that apparently did not express NKG2H. One possible solution to this conundrum was the idea that NKG2H triggering could result the induction of death of other T cells. To test the idea that the reduced proliferation of PBMCs after stimulation by anti-CD3/NKG2H antibody could be due to reduced cell survival, purified PBMCs were stimulated with immobilized anti-CD3 or anti-CD3/NKG2H antibodies for 48h and cell survival was measured by Annexin V/ 7AAD staining (Fig. 12-A).

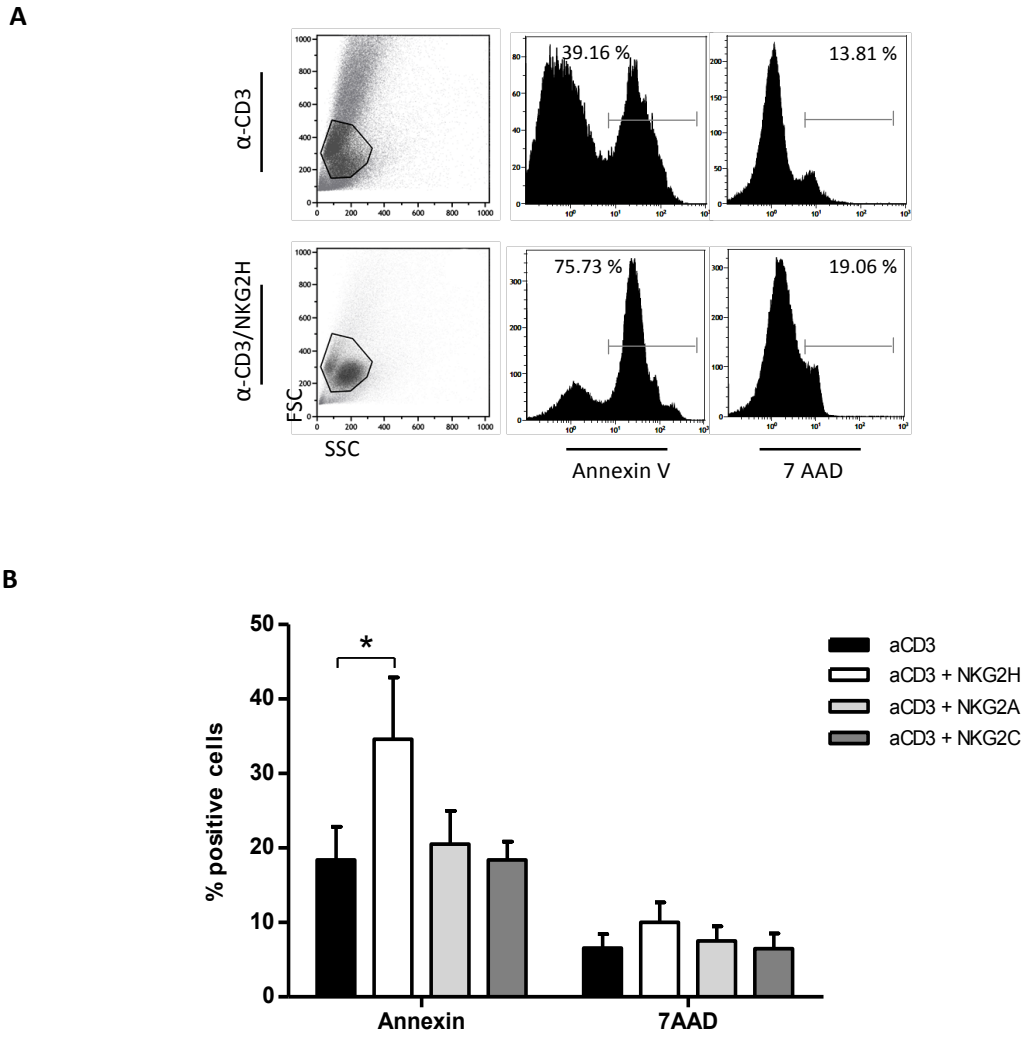


Fig 12: PBMCs cell survival decreases in cells stimulated by NKG2H, but not by NKG2A or NKG2C. Purified PBMCs were activated using immobilized anti-CD3 (1 μg/ml), and the combination of anti-CD3/NKG2A, anti-CD3/NKG2C or anti-CD3/NKG2H antibodies, at (1 μg/ml)/ (10 μg/ml) respectively for 48h. Cell survival was analysed by Annexin V/ 7AAD staining. **(A)** Representative dot plots of forward and sideward scatters and histograms are shown. **(B)** Changes in the percentage of cells positive for Annexin V and /7AAD was calculated and presented as graphic. Mean data and standard deviations are represented, n=7 in six independent experiments; *p<0.05. a/α – anti.

Stimulation of PBMCs with anti-CD3/NKG2A and anti-CD3/NKG2C antibodies was also carried out in parallel. Annexin V staining indicated a significant increase of cell apoptosis, when activation of PBMCs was done in the presence of anti-NKG2H stimuli. In contrast, no significant changes in cell apoptosis were observed in anti-NKG2A or anti-NKG2C stimulated PBMCs (Fig. 12-B).

3.5 NKG2H action does not depend on soluble factor.

The data outlined so far indicate that cultures stimulated with anti-NKG2H antibody showed reduced activation, a decreased proliferative response and an increased susceptibility to apoptosis. These multiple cellular functions could depend either on soluble factors or they could require direct cell contact.

To investigate the potential role of soluble factors released into the media after anti-NKG2H stimulation of PBMCs, supernatants collected from cell cultures after stimulation with either anti-CD3 or anti-CD3/NKG2H antibodies, were added at a range of different dilutions to a new set of cell cultures. No differences in the induction of expression of the activation marker CD69 were observed in any of the conditions used (Fig. 13), strongly suggesting that the release of soluble factors was not responsible for the NKG2H dependent reduction in cell activation and so, by elimination, cell to cell interaction is probably necessary for NKG2H to elicit the cellular effects shown previously.

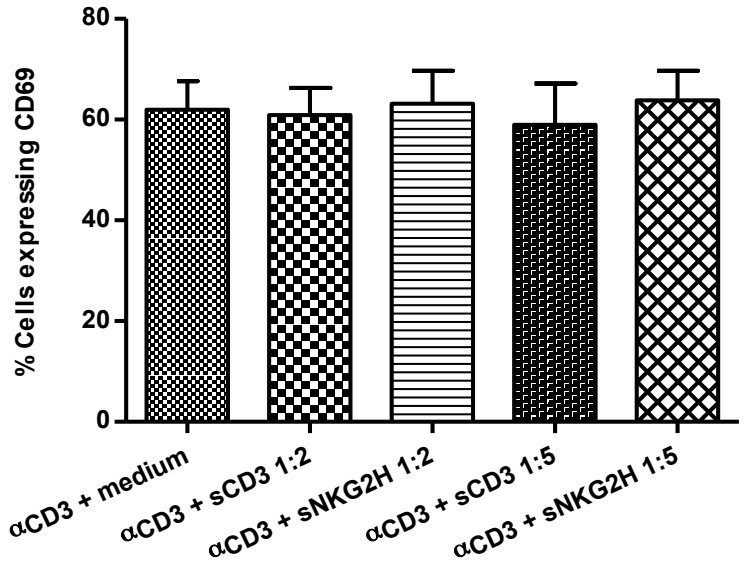


Fig. 13: Reduced cell activation induced by NKG2H stimulation is not mediated by a soluble factor. Paired sets of ¨donor¨ and ¨recipient¨ cell cultures were used for these studies as described in ¨Materials and Methods¨. Collected supernatants were used at 1:2 and 1:5 dilutions. Cell culture medium was used as a control. After 48h of incubation period with the supernatant, cells were harvested, stained for CD69 surface activation marker and checked the percentage of positive cells by flow cytometry. Mean data and standard deviations are represented, n=3 in three independent experiments. α– anti. s – supernatant.

3.6 NKG2H mediated activity is not MHC class I restricted.

As mentioned in the introduction MHC class I molecules are the major known ligands for the C-type lectin like CD94/NKG2 heterodimers. Therefore the possibility that MHC class I molecules could also be interacting with NKG2H was tested by including soluble MHC class I-specific blocking monoclonal antibody in the cultures where purified PBMCs were stimulated with either immobilized anti-CD3 or anti-CD3/NKG2H mAbs. Cell activation was assessed by staining for the surface marker CD69 after 48h stimulation of PBMCs with immobilized anti-CD3 or anti-CD3/NKG2H antibodies (Fig. 14).

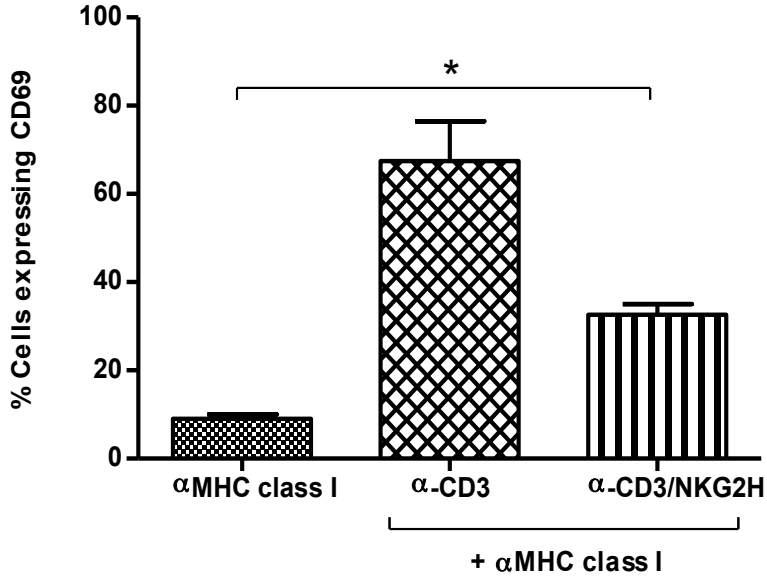


Fig 14: Antibodies to MHC class I did not recover the reduced cell activation seen after NKG2H stimulation. Freshly purified PBMCs were cultured over immobilized anti-CD3 (1 μg/ml) or anti-CD3/NKG2H [(1 μg/ml)/ (10 μg/ml)] antibodies for 48h in the presence of purified MHC class I (10 μg/ml) antibody. PBMCs cultured only in the presence of purified MHC class I antibody were used as a control. Mean data and standard deviations are represented, n=2 in one experiment.*$p<0.05$. α–anti.

The ability of the cells being cultured in the presence of NKG2H antibody to be activated at levels comparable with the control was not recovered, strongly indicating that MHC class I are not involved in the mechanism of action of NKG2H.

3.7 Strategies for the production of soluble CD94/NKG2H for use in ligand-hunting experiments.

In light of the previous experiments a key question was the identity of the molecule(s) with which NKG2H might interact. The strategy chosen to address this question was to try and produce soluble CD94/NKG2H (sCD94/NKG2H) protein that could be biotinylated and assembled into tetramers for use as a fluorescent reagent that would permit identification of cells and cell lines that were expressing NKG2H-binding proteins. Once cells, preferably tumour

cell lines, that express high levels of NKG2H ligands had been identified two possible strategies to molecularly characterize the NKG2H ligands could be followed: **1)** expression cloning using the fluorescent soluble NKG2H receptor preparations to identify and sort ligand expressing cells and **2)** the production of monoclonal antibodies that block the binding of soluble receptor to the immunizing cell line and that can then be used to biochemically characterize the putative ligand molecules for subsequently molecular or proteomic approaches, to definitively identify the NKG2H ligands. The laboratory had previously developed an *in vitro* system to express and refold sCD94/NKG2A and sCD94/NKG2C heterodimers (Vales-Gómez et al. 1999) and so the initial experiments aimed to reconstitute CD94/NKG2H receptor *in vitro* using purified, soluble protein produced in *Escherichia coli*. A construct directing expression of the extracellular portion of the NKG2H molecule modified to include an N-terminal target peptide for biotinylation by the BirA enzyme was designed, cloned and sequenced. However when attempts were made to express this sNKG2H protein in *E. coli* as inclusion bodies, no protein expression was observed. In contrast, the yield of sCD94 protein was as expected (Fig. 15).

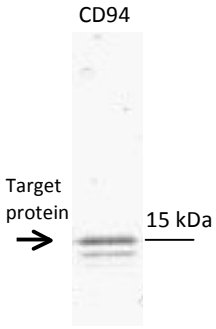


Fig. 15: SDS-PAGE analyzes of purified CD94 soluble protein. CD94 soluble protein purified from the inclusion bodies and solubilized in 8 M urea was analyzed by 12% separating gel (SDS-PAGE). In reducing conditions the CD94 protein appears as a 15 kDa band.

Systematic variation of the temperature of bacterial growth as well as changes in the concentration and time of IPTG induction or glucose supplementation of the growing media did not lead to any increase of protein yield. Similarly, modification of the N-terminal codons to use codons optimal for expression in E. coli also failed to increase NKG2H expression. Finally, attempts to isolate NKG2H-containing inclusion bodies from large-scale cultures of transformed bacteria (hoping to compensate for low yield per bacteria by increasing the numbers of bacteria in the culture) were also unsuccessful.

Given the failure to achieve expression of sNKG2H molecules in prokaryotic systems, a construct was prepared to express the extracellular portion of the NKG2H protein in a Baculovirus system. For these experiments we used plasmids designed to secrete soluble over-expressed proteins from the infected cell, gifts of Dr. F Rodriguez Aguirre (CNB). However, again, no success in expression of sNKG2H protein was achieved. These observations strongly suggest that factors intrinsic to the NKG2H sequence like mRNA secondary structure, limitations in the intrinsic ability of NKG2H to fold or a need for posttranslational modifications may negatively influence expression of this gene.

Part II. Characterization of *M. tuberculosis* – peptide specific HLA-E restricted T cells in bladder cancer patients receiving BCG immunotherapy

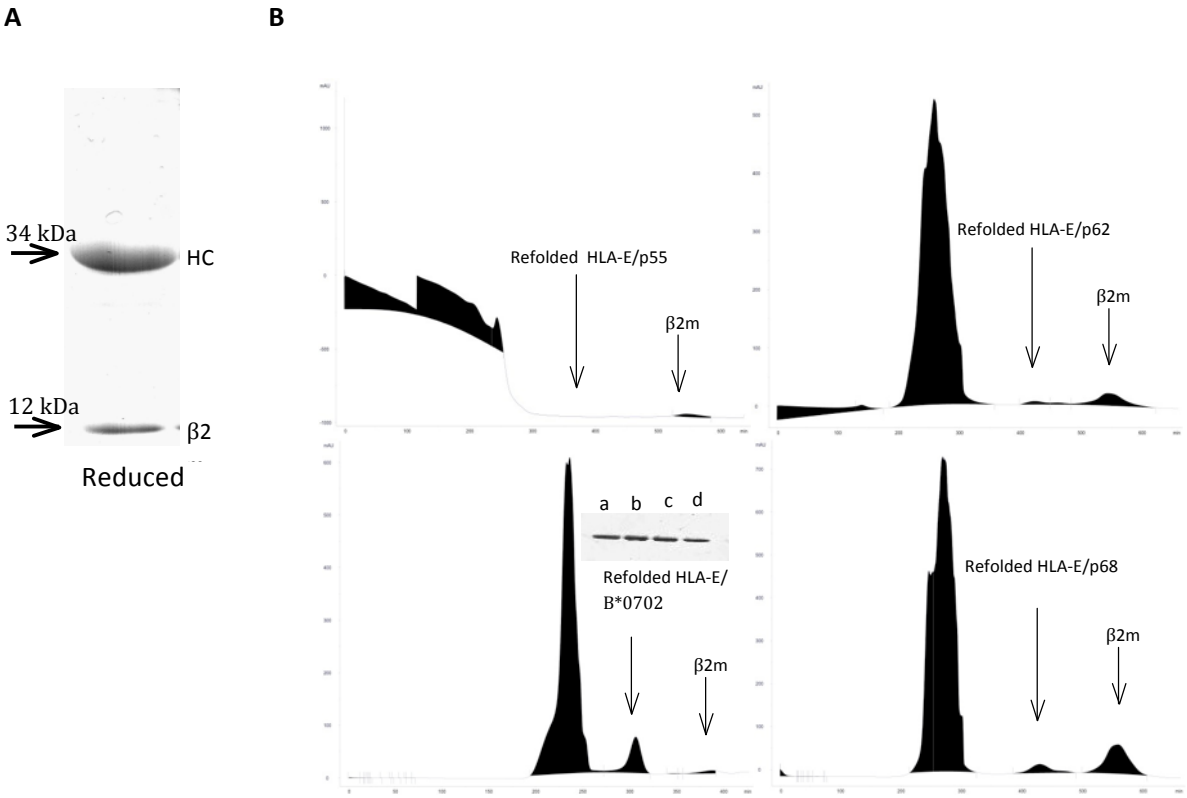
Since 1976, immunotherapy with live bacilli Calmette Guerin (BCG) has proved to be an effective adjuvant intravesical treatment to prevent progress and relapse after transurethral resection of non-muscle invasive bladder cancer (Morales et al. 1976; Sylvester et al. 2002; Herr et al. 2008). The antitumor effect of the BCG therapy is characterized by the induction of a strong innate immune response (Higuchi et al. 2009) as well as T cell infiltration into the bladder (Bohle et al. 2003; Brandau et al. 2007; Cooper et al. 2009). One aspect of this therapy that has proved controversial is whether immunity to tuberculosis antigens, is of benefit to the patient. Studies evaluating how the appearance of PPD-reactive T cells after intravesical therapy correlates with clinical outcome, have given mixed results, including **1)** a positive correlation with clinical response (Kelley et al. 1986; Torrence et al. 1988; Lamm et al. 1991), **2)** a weak association (Lüftenegger et al. 1996) or **3)** no correlation whatsoever (Shinka et al. 1990; Bilen et al. 2003). In agreement with the last set of studies, clinical trials to investigate the combined use of intravesical and intradermal (the normal route of BCG vaccination) exposure to BCG showed no evidence that an enhanced reactivity to PPD was associated with a better clinical response in bladder cancer (Lamm et al. 1991; Lüftenegger et al. 1996). However, recently it has been suggested that parenteral immunization with BCG to generate systemic immunity, before initiating intravesical instillations could be associated with an improved response to therapy (Biot et al. 2012).

In view of this contradicting data in the literature, it was of interest to revisit this issue taking advantage of new knowledge and using newer technologies that allow a more detailed characterization of *M. tuberculosis* – specific $CD8^+$ T cells induced after BCG instillation therapy in patients with bladder cancer. Assessment of T cell functionality by cytokine profiling, in addition to the more than 100-years-old tuberculin skin test has been applied to indicate pre-exposure to Mtb as well as to distinguish patients with and without active disease (Millington et al. 2007; Caccamo et al. 2010; Day et al. 2011; Harari et al. 2011; Sester et al. 2011; Petruccioli et al. 2013). Additionally, well-defined peptide epitopes and tetramers have already been used for detection of Mtb specific T cells in PBMCs and also directly in situ, in granuloma tissue (Geluk et al. 2000; Tully et al. 2005; Hohn et al. 2007; Caccamo et al. 2009). Recently, this finding has also been combined with IFN-γ and TNF-α detection showing that at least some antigen-specific Mtb-specific T cells produced IFN-γ and/or TNF-α (Li et al. 2011) and suggests that these tetramer positive cells may be functional (Bronke et al. 2005).

Most of the MHC class I tetramers used in the experiments mentioned above are complexes of HLA-A*02:01 molecules and specific – epitopes, derived from Mtb, that bind to this HLA allele. However, the non-classical HLA-E protein has been also shown to present peptides derived from Mtb, leading to expansion of HLA-E restricted $CD8^+$ T cell clones (Heinzel et al. 2002). Thus, it seemed reasonable to hypothesize that the use of tetramers comprising of HLA-E loaded with Mtb-derived peptide epitopes could be a simple approach to detect Mtb-reactive T cells in a wide variety of patients given that HLA-E displays only very limited polymorphism. This

approach was made possible by the characterization of a large panel of Mtb derived peptides presented by HLA-E molecules and recognized by $CD8^+$ T cells by Joosten et al. at 2010.

To generate tetrameric complexes using peptides epitope reported to have a high binding affinity for HLA-E, three peptides, p55 (VMATRRNVL) (HLA-E/p55), p62 (RMPPLGHEL) (HLA-E/p62) and p68 (VLRPGGHFL) (HLA-E/p68) were selected and refolded with HLA-E *in vitro* for subsequent assembly into fluorescent tetramer complexes (Fig. 16-B and Table 1). We also prepared a control HLA-E tetramer, using a nonameric peptide derived from the signal sequence of HLA-B*0702 (HLA-E/B*07), that is known to strongly bind and induce surface expression of HLA-E (Fig. 16 and Table 1) (Braud et al. 1997; Borrego et al. 1998; Braud et al. 1998[(1)]; Lee et al. 1998[(1)(2)]; Vales-Gomez et al.1999). Unfortunately, although the p55 peptide was reported to be recognized by T cells from Mtb-exposed donors in an HLA-E restricted manner this peptide did not refold HLA-E efficiently and so reactivity with this peptide/HLA-E complex could not be studied further. Once generated, these tetramers were first tested for specific binding using whole blood from healthy individuals immunized or not with Bacillus Calmette Guerin (BCG) vaccine and later tetramer reactivity with PBMCs from 9 bladder cancer patients who received BCG-instillation therapy (mean age 72 years) was studied.



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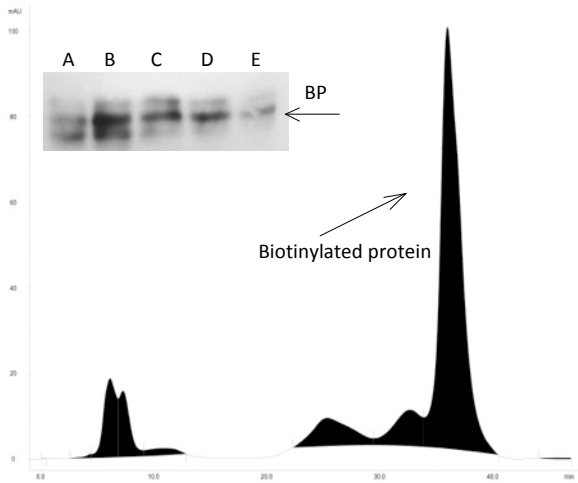


Table 1. Summary of the purification steps and final yield of HLA-E refolded monomers

HLA-E /Peptide sequence	Size-exclusion Chromatography	Ion-exchange Chromatography	Refolded monomer (mg)
	Peak Area /mAU*min	Peak Area /mAU*min	
VMAPRTVLL (HLA-B*0702)	25425	216	0.7
VLRPGGHFL (*Mtb*-p62)	182	82	0.18
RMPPLGHEL (*Mtb*-p68)	1004	58	0.2
VMATRRNVL (*Mtb*-p55)	/	/	/

Fig. 16: SDS-PAGE analyses of recombinant HLA-E soluble protein after refolding in vitro (A). HLA-E soluble protein refolded with HLA-B*0702 peptide was analysed by 12% SDS-PAGE before protein purification by size exclusion chromatography. SDS-PAGE was run in reducing conditions. The position and the size of the heavy chain (HC) and β2m are indicated. **(B and C) Purification of HLA-E/p55, HLA-E/p62, HLA-E/p68 and HLA-E/ B*0702 recombinant proteins. (B)** Elution profile obtained from size-exclusion chromatography step using a Sephacryl S-300 column is presented. The mobility of aggregated material, refolded HLA-E and free β2m is shown. Inset: SDS-PAGE analyses showing relevant peak fractions. (a-d)–peak fractions **(C)** Representative elution profile from purification of biotinylated refolded proteins by ion-exchange chromatography using HiTrap Q Sepharose FF column. Inset: western blot analyses of purified protein-biotin peak fractions visualized using Avidin-HRP. BP- biotinylated recombinant protein. (A-E) – peak fraction **Table 1.** Summary of the purification steps and final yield of HLA-E refolded monomers.

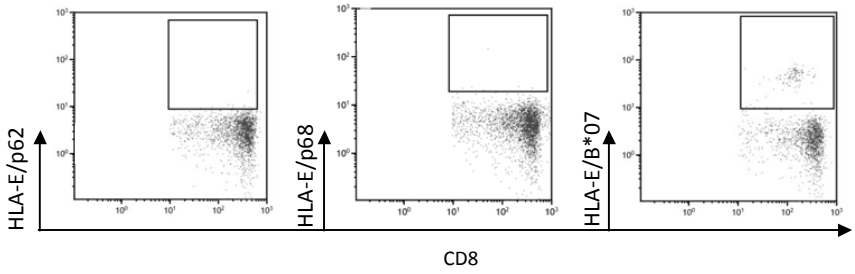
1. *M. tuberculosis* specific CD8+ T cells in healthy individuals immunized or not with BCG.

Bacillus Calmette-Guerin (BCG) is a live vaccine derived from a strain of *Mycobacterium bovis* that was attenuated by Calmette and Guerin at the Pasteur Institute after passaging for 12 years and was first administrated to humans in 1921 (Grange et al. 1983). Although all the currently used vaccines are derived from the original BCG strain, they differ in their characteristics when grown in culture and in their ability to induce an immune response to tuberculin.

The possible existence of Mtb-primed T cells in healthy people being vaccinated with BCG should have allowed us to test for reactivity with HLA-E tetramers loaded with *M. tuberculosis* peptides p62 and p68. We collected blood samples from 6 adult volunteers who had been vaccinated with BCG in the first year of life. The average time between last vaccination and the blood test was 27 years. A group of 3 healthy volunteers with no history of exposure to BCG or *M. tuberculosis* infection acted as controls.

Flow cytometry staining of whole blood samples using HLA-E/p62 -, HLA-E/p68 - tetramers did not detect BCG-specific $CD8^+$ T cells in any of the healthy donors. Nevertheless, $CD8^+$ T cells reactive with the HLA-E- HLA-B*0702 peptide could be observed in one of the examined donors (Fig. 17-A). This likely reflects expression of CD94/NKG2A heterodimers by a subpopulation of $CD8^+$ T cells in this donor. However, the HLA-E/B*07 tetramer stained NK cells in all donors tested.

A



B

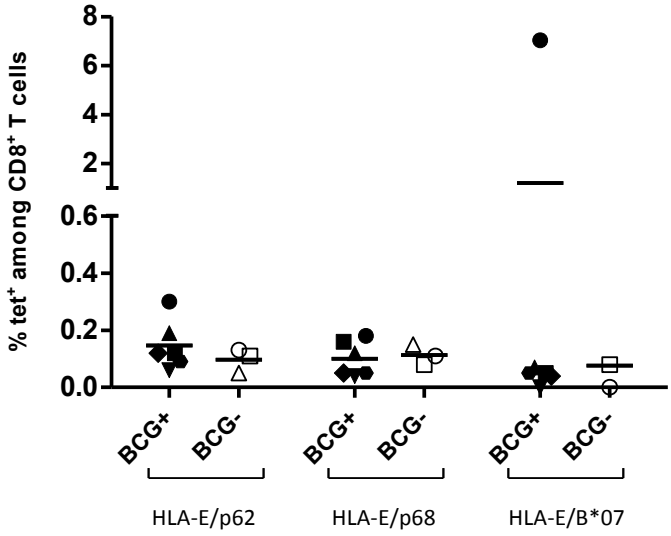


Fig. 17: Screening of Mycobacterium tuberculosis specific CD8+ T cells in blood from healthy volunteers previously exposed or not to the pathogen using tetrameric complexes. (A) Representative flow cytometry dot plots for tetramer staining of PBMCs. **(B)** Quantification of the percentage of tetramer+ cells among CD8+ T cells found in peripheral blood from healthy BCG no/vaccinated donors. Each symbol corresponds to the result obtained from an individual donor. Mean data are represented, n=6 in one experiment..

The percentages of HLA-E/p62 -, HLA-E/p68 - and HLA-E/B*07 tetramer positive cells among the $CD8^+$ T cells between different groups of donors did not show any significant difference (Fig. 17-B). This result was not entirely unexpected as numerous results obtained in controlled clinical trials have shown that BCG driven protective immunity diminishes with the age and a booster tuberculosis BCG vaccine is needed to induce measurable protection against virulent bacteria in adults vaccinated with BCG when neonates (Mangtani et al. 2013). However this result meant that there was no positive control for staining of PBMCs using these tetramers. To try and increase the frequency of BCG reactive T cells before staining, PBMC from a vaccinated individual were restimulated with live BCG for 5 and 12 days in culture, before staining with the teramers, but again no convincingly positive population was identified.

2. Screening for tetramer$^+$ cells among T cell in blood from patients with bladder cancer receiving instillations of live BCG

Simultaneous with the above experiments a group of nine patients with bladder cancer receiving BCG therapy was screened for the existence of Mtb-peptide specific HLA-E restricted T cells using HLA-E/p62 - and HLA-E/p68 - tetramers. The characteristics of the patients included in this study are presented in Table 1 (Materials and Methods). Samples of peripheral blood, collected before each instillation as indicated in Material and Methods, were analysed by flow cytometry for the presence of tetramer reactive T cell populations. Tetramer$^+$ cells could be detected at frequencies as low as 0.01% of gated cells. The observed range of tetramer percentage values in this study was 0.03-0.22% (mean 0.08±0.05) for HLA-E/p62-Tet^+ $CD8^+$cells, 0.01-0.07% (mean 0.04±0.04) for HLA-E/p68-tet^+ $CD8^+$ cells and 0.5-8.7% (mean 2.3±2.8) for HLA-E/B*07 -tet^+ $CD8^+$ cells. When the percentage of Mtb Tet^+ $CD8^+$ T cells was analysed in detail, little correlation between the frequencies of tetramer$^+$ cells at different patients at the same time point of BCG instillations was found (Fig. 18). Further, no correlation between Mtb tetramer$^+$ cells and the time points of instillation within the course of treatment of a single patient was observed. However, in some of the patients a small increase of the HLA-E restricted T cells could be observed during the resting period, between the two courses of BCG instillations. The percentage of these cells tends to drop again between two BCG instillations in the same course. This observation might be explained if activated, HLA-E restricted T cells infiltrate into the bladder after intravesical instillations of live BCG and so only a very low number of these cells can be found circulating in the blood stream. Previous investigations have suggested that for robust T cells infiltration of the bladder wall repeated instillations with live BCG were necessary (Biot et al. 2012). This might also explain the observed

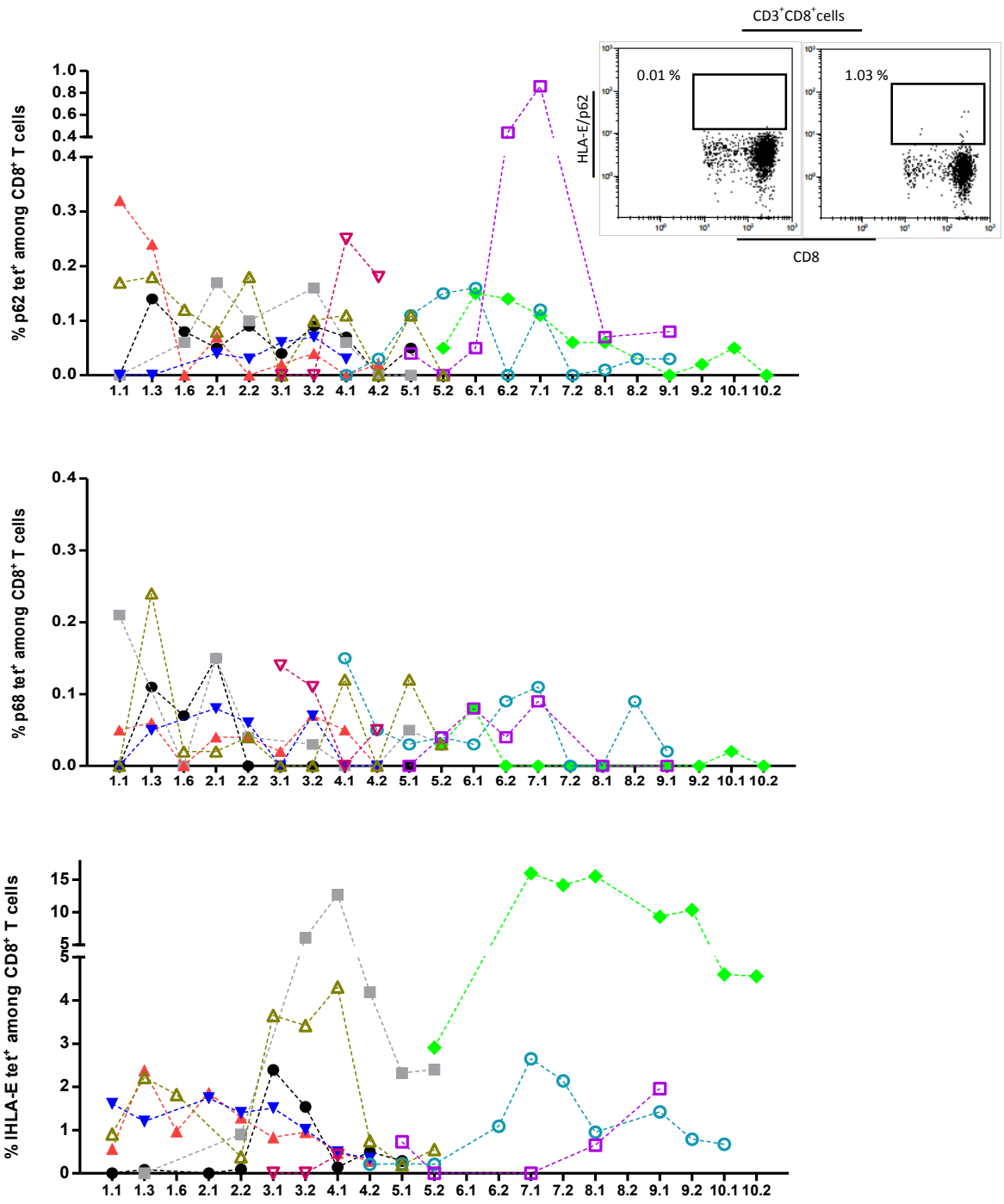


Fig. 18: Plots showing the variations over time in the percentages of HLA-E/p62 tet⁺ cells, HLA-E/p68 tet⁺ cells and HLA-E/B*07 tet⁺ cells among CD8⁺ T cells in bladder cancer patients receiving therapy with live BCG. Whole blood samples from the patients were stained as indicated in ¨Material and Methods¨. For each individual donor the percentage of tetramer⁺CD8⁺cells was assessed at the different time points. Numbers in the X-axis indicate the different BCG instillations. The blood sample was collected from the patients before each instillation. Each symbol corresponds to one individual donor. Inset: Representative flow cytometry dot plot of the HLA-E/p62 tet⁺ CD8⁺ T cells in one of the patients showing expansion of these cells at B6.1 and B7.1 time points.

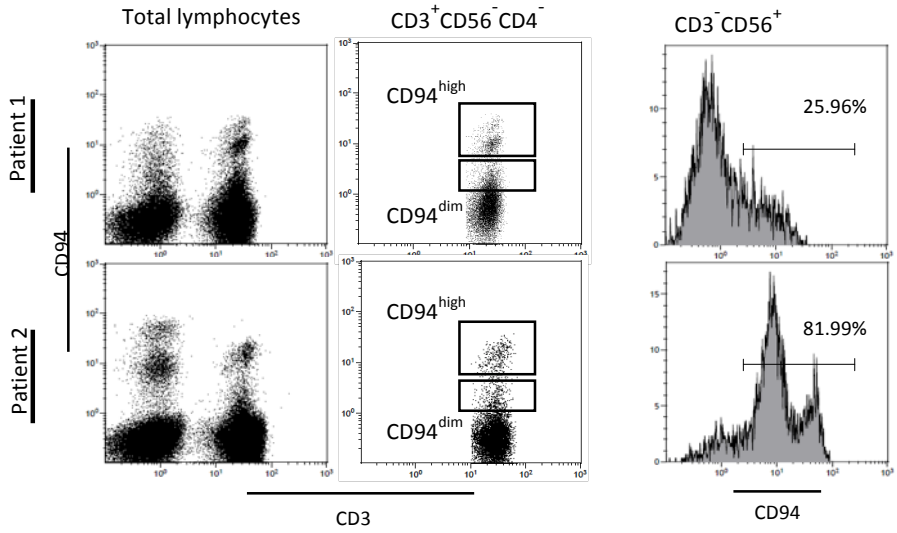
variation in the frequency of HLA-E restricted T cells found in the blood stream of the patients at the beginning of the anti-bladder cancer BCG treatment. Importantly, the pre-existing cellular immunity towards PPD for the patients included in this study is unknown. Thus it is also possible that the tetramer^{+} cells detected in the blood of some of the patients before receiving their first instillation of BCG are memory T cells generated during prior exposure to Bacillus Calmette Guerin (BCG) vaccine or M. tuberculosis infection. Another possibility is that although the HLA-E binding peptide epitopes used in this study were chosen because they were commonly recognized by a panel of Mtb exposed donors (Joosten et al. 2010), it could be that the pattern of epitopes recognized after intravesical instillation is different, or simply that although HLA-E restricted CD8^{+} T cell responses are found in Mtb infection, these responses are not generated efficiently in these bladder cancer patients. Again perhaps, the route of immunization may influence the nature of the immune response made to BCG in these patients. It should also be remembered that there was no positive control for the staining with the Mtb tetramers and so the possibility that the lack of staining of the patient samples reflects some problem (e.g. stability or folding) with these tetramers cannot be excluded.

Finally it is also interesting to note that expansions of CD8^{+} T cells reactive with the HLA-E/B*07 - tetramer are observed in some patients during the course of treatment. Previous data (Valés-Gómez et al. 1999) showed that although HLA-E molecules loaded with the HLA-B*0702 peptide bind both CD94/NKG2A and CD94/NKG2C receptor, the binding to the CD94/NKG2A heterodimer is of markedly higher affinity. Thus it seems reasonable to suggest that the HLA-E/B*07 tetramer staining observed most probably corresponds to CD8^{+} T cells that express inhibitory CD94/NKG2A receptors.

CD8^{+} T cells that express CD94/NKG2A receptors generally correspond to a subset of CD8^{+} T cells with an activated/memory (CD44^{hi}) phenotype (Braud et al. 2003). Although naive CD8^{+} T cells can express CD94/NKG2A receptors after TCR engagement (McMahon et al. 2002; Moser et al. 2002; Wojtasiak et al. 2004; Zhou et al. 2008) and CD94/NKG2A expression can be modulated by cytokine treatment: incubation with IL-21 triggers CD94/NKG2A up-regulation, whereas IL-4 or TGF-beta leads to receptor down-regulation (Brady et al. 2004; Gays et al. 2005), TCR interaction seems to be a prerequisite for induction of expression of CD94/NKG2A by T cells *in vivo*.

In parallel to this work, other members of the laboratory have studied the distribution of a range of different markers expressed on the cell surface of leukocytes circulating in the blood of these patients. Thus, we analysed the changes in the expression of the CD94 receptor in the blood of the studied bladder cancer patients and tried to correlate these data to the observed HLA-E/B*07 – tetramer^{+} staining of CD8^{+} T cells. This analysis has proved to be complicated since not only the percentage of CD94^{+} cells varied widely between the different patients but also these patients expressed distinct subsets of T cells, as defined by their CD94 surface levels (CD94^{+}, CD94^{dim}, CD94^{high+}, CD94^{high++}) (Fig. 19-A). Moreover, for technical reasons, the cocktail of antibodies used for staining patient blood that included CD94, did not contain CD8 and this obviously further complicated comparison of the staining experiments using HLA-E/B*07 tetramers and CD94 mAb.

A


Total lymphocytes
CD3+CD56-CD4-
CD3-CD56+
Patient 1
Patient 2
CD94
CD94high
CD94dim
25.96%
81.99%
CD3
CD94

B

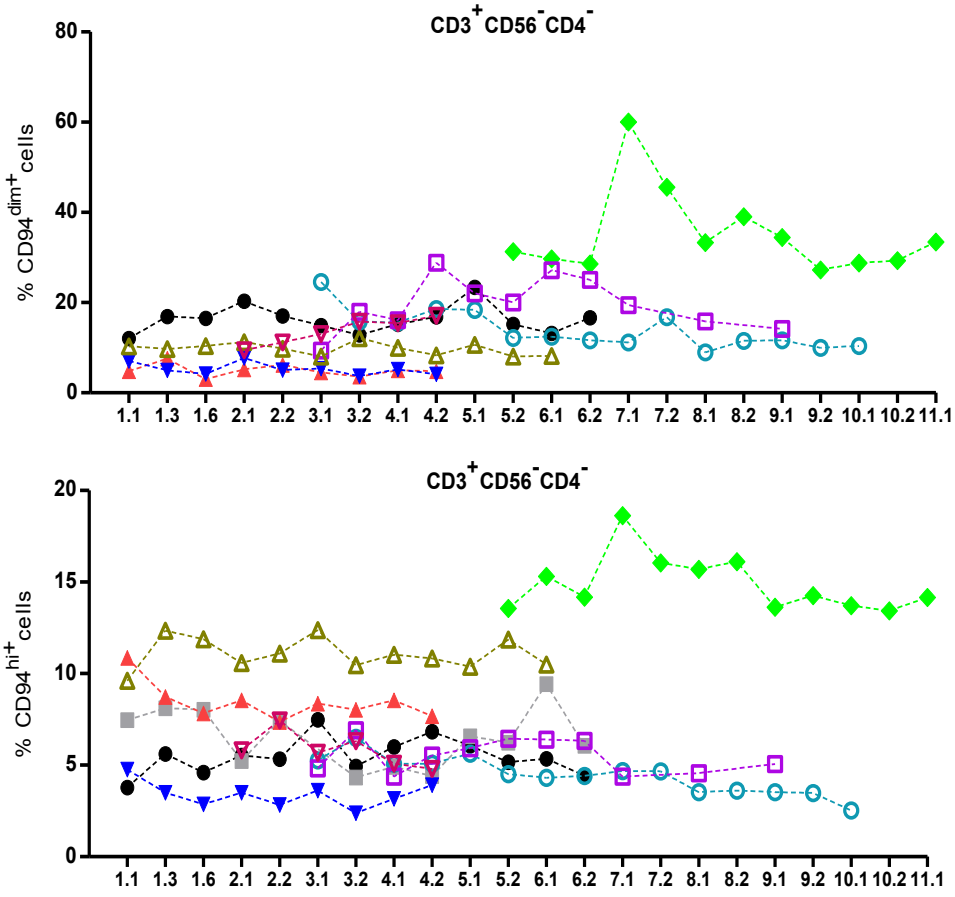

CD3+CD56-CD4-
% CD94dim+ cells
CD3+CD56-CD4-
% CD94hi+ cells
1.1 1.3 1.6 2.1 2.2 3.1 3.2 4.1 4.2 5.1 5.2 6.1 6.2 7.1 7.2 8.1 8.2 9.1 9.2 10.1 10.2 11.1

Fig. 19: Expression of the CD94 receptor on $CD3^+CD56^-CD4^-$ T cells in bladder cancer patients receiving BCG immunotherapy. (A) Representative flow cytometry dot plots and histograms showing the different patterns of expression of the CD94 receptor in the blood of two bladder cancer patients undergoing BCG treatment. **(B)** Whole blood samples from the patients were stained using combination of the following antibodies: anti-CD3-FITC, anti-CD56-PE, anti-CD4-PerCP/Cy5.5 and CD94-APC. For every individual donor the percentage of $CD94^{dim+}$ and $CD94^{hi+}$ cells among $CD3^+CD56^-CD4^-$ cells was assessed across the different time points. Numbers in the X-axis indicate the time points of each BCG instillation. The blood sample was collected from the patients before each instillation. Each symbol corresponds to the result obtained from an individual donor.

Two NK cell subsets, defined by their level of CD94 expression, were described by Perez-Villar et al. 1996; NK cells with high levels of CD94 corresponded to CD94/NKG2A expressing NK cells and cells with low levels of CD94 were enriched for CD94/$NKG2C^+$ NK cells. Similarly, T cells with different levels of CD94 expression have also been described (Jabri et al. 2002). NKG2A transcripts were specifically found in $CD94^{high}$ $\alpha\beta^+$ T cells, but this population also expressed NKG2C and NKG2E (or NKG2H, the oligonucleotides used for PCR would not distinguish between NKG2E and NKG2H). $CD94^{dim}$ T cells expressed NKG2C and/or NKG2E/NKG2H, but not NKG2A transcripts. There are also some $NKG2^-$ T cell clones expressing CD94 homodimers (Jabri et al. 2002). As described for NK cells, redirected lysis assays, where the TCR was co-crosslinked with CD94, demonstrated that $CD94^+$ T cells could be divided into three functionally distinct subsets based on the expression of inhibitory $CD94^{high}$ NKG2A receptors, activating $CD94^{low}$ NKG2C/E receptors, or non-functional CD94 homodimers. CD94 engagement in clones expressing both inhibitory NKG2A and activating NKG2C, E resulted in TCR inhibition, thus the inhibitory phenotype appears to be dominant. Analyses of the TCR repertoire expressed by these clones revealed that commitment to inhibitory NKG2A expression was an attribute acquired after TCR expression and during antigen encounter, whereas actual surface expression depended on recent TCR engagement. CTL clones expressing sequence-related TCR, and so probably specific for the same antigens, invariably shared the same NKG2A commitment suggesting that TCR antigenic specificity dictates NKG2A commitment, which critically regulates subsequent activation of CTL (Jabri et al. 2002).

In the experiments staining bladder cancer patients, some of the expansions of $CD8^+$ T cells reactive with the HLA-E/B*07 - tetramer could be correlated with the change of the cell surface expression of $CD94^{high+}CD3^+CD56^-CD4^-$ cells seen in some of the patients and so could illustrate change of the levels of $NKG2A^+$ T cells (Fig. 19-B). However, in one of the patients the HLA-E/B*07 - tetramer staining displayed the same pattern of recognition of $CD94^{high+}CD3^+CD56^-CD4^-$ ($NKG2A^+$) as for $CD94^{dim+}CD3^+CD56^-CD4^-$ ($NKG2C^+$) T cells. This could mean that NKG2C receptor expressed on T cells in this patient is not only expressed in higher percentage compared to the other patients but also has an increased affinity for HLA-E/B*07 ligand. Alternatively, this staining could be due to CD94/NKG2A receptor expression that, for some reason (e.g. cytokine modulation), is dull on the T cells of this individual at this time point. These analyses are very complex, since $CD94^{high}$ from $CD94^{dim}$ subpopulation could not be separated clearly in each patient. Moreover, in some patients the existence of $CD94^{high++}$cell subpopulation as well as changes in the percentages of these cells varied during the time

course of the instillations (Fig. 19-A) (data not shown). As these results are from a study currently underway, it would be interesting, and feasible, to modify future analyses to include more markers like anti-NKG2A/NKG2C/NKG2H antibodies to more fully characterise the CD94 expressing T cell subpopulations.

The significance of induced expression of immunoregulatory molecules such as CD94/NKG2A on $CD8^+$ T cells is not fully understood, but it is generally thought that these receptors restrain $CD8^+$ responses, so preventing T-cell exhaustion in chronic infections and limiting immunopathology. NKG2 -receptors has also been implicated in the cell survival of both NK and CD8 T cells. It was found that the extent of apoptosis in $CD8^+$ T and NK cells was inversely related to the expression of CD94, with lower levels of apoptosis seen in $CD94^{high}$ cells after 1-3 days of culture. This correlation between CD94/NKG2 expression and lower levels of apoptosis may reflect an important role of these receptors in the maintenance of $CD8^+$ T and NK cells (Lohwasser et al. 2001; Gunturi et al. 2003; Gunturi et al. 2004; Gupta et al. 2012). Moreover, as mentioned above, expression of CD94/NKG2A heterodimers at the cell surface reflects recent TCR engagement (Jabri et al. 2002), thus the HLA-E/B*07 - tetramer staining may be identifying recently stimulated T cells. In the context of these cancer patients it is not currently possible to know whether this expression of CD94/NKG2 receptors is associated with a net positive or negative effect, but as the study progresses it will be interesting to compare the clinical outcome of those patients that have experienced CD94/NKG2A/NKG2C/NKG2H upregulation on $CD8^+$ T cells with those who have not.

Discussion

CD94/NKG2H C-type lectin like receptor complex and HCMV infection

NKG2H, a splice variant of NKG2E was described by Bellon et al. at 1999. However, to our knowledge, no follow-up studies have been published, presumably the lack of a specific monoclonal antibody is the main reason that has severely restricted progress in understanding the immunological function of this receptor. In this work using a newly commercially available anti-NKG2H monoclonal antibody, the phenotypic properties and to some extent the functional capacity of lymphocytes expressing the NKG2H receptor has been studied and defined.

NKG2H was found to be expressed at low levels on the surface of a small fraction of PBMCs derived from healthy donors. Although some expression of NKG2H was detected on NK cells and $CD4^+$ T cells, this molecule was preferentially expressed on $CD3^+CD56^+$ and $CD3^+CD8^+$ cells implying that the signals controlling the expression of NKG2H receptor on the cell surface do not operate equally in NK and T cell subsets. Interestingly, NKG2H protein was first identified and the cDNA obtained from a human TCR$\alpha\beta$ $CD8^+$ T cell clone (TCC) (Bellón et al. 1999). Moreover, both CD94/NKG2A and CD94/NKG2C NK cell heterodimeric receptors have been described to be expressed and functional in $\alpha\beta$ and $\gamma\delta$ $CD8^+$ T cells populations (Poccia et al. 1997; Noppen et al. 1998; Uhrberg et al. 2001; Jabri et al. 2002; Guma et al. 2005; Moser et al. 2002; Meresse et al. 2006). In $\alpha\beta$ T cells the expression of NKG2A and NKG2C is different, so that CD94/NKG2C is expressed by highly differentiated $CD8^+$ effector T cells whereas CD94/NKG2A is present on effector cells as well as on memory cells (Arletazz et al. 2004). Additionally, it has been shown that both types of receptors are expressed at different moments during $\gamma\delta$ T cell effector differentiation and function (Angelini et al. 2010). It remains to be defined whether NKG2H molecule expression is stochastic or restricted to naive, memory or some effector subset of $\alpha\beta$ and/or $\gamma\delta$ T cells, and if CD94/NKG2H heterodimer can be co-expressed or not on these cells with the other members of this receptor family. One note of caution that should be borne in mind for these experiments is that currently only one mAb specific for NKG2H has been described. This mAb was raised against a peptide sequence that is specific for NKG2H (corresponds to the last 23 amino acids of the extracellular domain; TBLASTN search 22nd July 2014) and as has been shown by the manufacturer, (http://www.rndsystems.com/ScientificPoster_nkg1_family.aspx) does not stain NKG2A or NKG2C transfectants. This observation was confirmed using RBL cell transfected with CD94, CD94/NKG2A and CD94/NKG2C (gifts of Miguel López-Botet, UPF, Barcelona) before beginning the experiments presented in this thesis (data not shown).

Evidence is accumulating that indicates that HCMV infection is associated with a stable reshaping of the repertoire of receptors expressed by NK cells, leading to a variable expansion of $NKG2C^+$ NK and T cell subsets in HCMV seropositive healthy individuals (Guma et al. 2004) and aviremic HIV-1 infected patients (Guma et al. 2006[2]; Mela et al. 2007; Muntasell et al. 2013[1]; Lopez-Botet et al. 2014). The structural similarity between NKG2H and NKG2C suggested the possibility that HCMV infection may also influence NKG2H expression by the cells present in the PBMC. To address this idea, we stimulated purified PBMCs with HCMV infected fibroblasts; an

experimental system previously employed to study virus-specific CTLs (Walter et al. 1995) and already used to study NKG2C$^+$ cells in HCMV infection (Guma et al. 2006). Experiments done with freshly isolated PBMCs from blood of healthy volunteers showed **an increase in the proportion of NKG2H$^+$ cells** after co-culture of the PBMCs with HCMV infected fibroblasts similar to that observed for NKG2C$^+$ cells. These data have been generated in an allogeneic environment; however no changes in NKG2H receptor expression on control PBMCs incubated with mock infected fibroblasts was observed. Further, the expansion of NKG2C$^+$ cells after co-culture of PBMC from HCMV seropositive individuals with HCMV infected fibroblasts has previously been shown to be comparable in allogeneic and autologous systems (Guma et al. 2006; Petersen et al. 2010). To what extent the CD94/NKG2H heterodimer is involved in driving the observed HCMV induced T cell and NK cell expansion constitutes a relevant question. In PBMCs from healthy donors the NKG2H receptor was expressed at relatively modest levels, preferentially detected on CD8$^+$ T cells and CD3$^+$CD56$^+$ double positive cells but only a minor proportion on NK cells. During the period of co-culture of PBMCs with HCMV infected fibroblasts an increase of the percentage of NKG2H expressing T cells, CD3$^+$CD56$^+$ cells as well as NK cells was noted- weak after three days and much more prominent after 7 days of culture. These data are easier to reconcile with a model where the cells have acquired NKG2H receptor after exposure of the PBMCs to virus infection rather than that the NKG2H$^+$ cells have expanded. However, NKG2H specific expansion during HCMV infections cannot be excluded. Indeed the transwell experiments (Fig. 5) indicate that contact between the virus-infected cells and the lymphocytes markedly enhances the increase in NKG2H expression, although this could also reflect enhanced lymphocyte activation due to cell:cell contact.

In parallel to the analysis of NKG2H expression, changes in the proportion of NKG2C and NKG2A molecules on PBMCs after stimulation with HCMV infected fibroblasts were also examined. Whereas the expansion of NKG2H$^+$ cells was consistent over multiple experiments no consistent changes in the frequency of NKG2C and NKG2A expressing cells were observed. According to the published data, NKG2C$^+$ cell expansion in vitro after exposure to HCMV infected cells is associated with positive serology for HCMV so that it appears in HCMV-seropositive donors exclusively. As the majority of our healthy donors were HCMV-seronegative, the lack of change in the proportion of NKG2C$^+$ cells in these samples was to be expected. In some studies, where the co-culture protocol was essentially identical to our experiments, no differences in the expression of the NKG2A receptor on PBMCs upon co-culture with mock or HCMV infected fibroblasts were described and the frequency of NKG2A$^+$ cells appeared not to be related to HCMV seropositivity status. (Guma et al. 2006; Lopez-Verges et al. 2011; Wu et al. 2013). One study has described an increase of functionally active CD94/NKG2A$^+$ NK cells that occurs after 24 and 72 hours of addition of PBMCs to HCMV infected fibroblasts, but an important difference between those experiments and the data presented in this thesis is that these fibroblasts were in late stages of the viral replication since they had been cultured for 4 days after the initial infection before the addition of PBMCs (Petersen et al. 2010).

The molecular mechanisms underlying the induction of NKG2H after contact with HCMV-infected fibroblasts are not clear. CD94/NKG2A expression has been previously shown to be induced in T cells stimulated with IL -12 (Derre et al. 2002) or upon TCR dependent activation under the influence of IL-15 or TGFβ (Mingari et al. 1998; Bertone et al. 1999). Virus infection can also induce the production of IL-15 (Ahmad et al. 2000; Azimi et al. 2000), a cytokine that plays a major role in regulating NK cell proliferation and differentiation, contributing to the accumulation of NK cells in CMV-infected mice (Nguyen et al. 2002). Moreover, an increased proportion of $NKG2A^+$ lymphocytes were observed in NK cell populations cultured in the presence of IL-12 (Draghi et al. 2005), whereas endogenous IL-12 secretion in HCMV-infected monocyte-derived dendritic cells (moDC) cultures has been described to induce NKG2A expression in $NKG2C^+$ cells (Saez-Borderias et al. 2009). The transwell cell culture experiment (Fig. 5) where PBMCs were cultured either in contact with HCMV infected fibroblasts or in different chambers where only diffusion of soluble factors was permitted indicated that although signals in the form of soluble factors induced a small increase in the percentage of $NKG2H^+$ cells, contact between PBMCs and HCMV infected fibroblast was required for efficient expansion of $CD94/NKG2H^+$ cells. Altogether, the observations suggest that HCMV-induced changes in the cytokine network may contribute but are not sufficient to strongly drive increased NKG2H expression by lymphocytes in these cultures. The contact dependence of this phenomenon could suggest the possibility of a putative ligand expressed by HCMV-infected fibroblasts able to engage the CD94/NKG2H receptor. A peptide from the leader sequence of the UL40 HCMV protein is known to preserve HLA-E expression in HCMV-infected fibroblasts, protecting them from lysis by $CD94/NKG2A^+$ NK cells (Tomasec et al. 2000; Ulbrecht et al. 2000; Wang et al. 2002). Polymorphisms of HCMV UL40 have been shown to impact the affinity of the interaction with the inhibitory CD94/NKG2A or activating CD94/NKG2C receptors on NK cells (Heatley et al. 2013). Thus it is at least conceivable that some viral protein, or peptide from a viral protein presented in the context of an HLA molecule, could contribute to the increased NKG2H expression by T and NK cells in these cultures. Another possibility is that the NKG2H receptor could react with molecular patterns presented on infected cells, which are not restricted/associated with HLA. A final possibility is that NKG2H expression is a secondary consequence of activation of some populations of lymphocytes, however this model does not explain why only some strains of HCMV drive an increase in NKG2H expression.

Each clinical HCMV strain isolated has the potential for a unique identity (Kilpatrick et al. 1976; Huang et al. 1976), which leads to the hypothesis that strain variation, affects the outcome of HCMV infection (Cerboni et al. 2000). Most genes are highly conserved in sequence between HCMV strains, but a number of genes predicted to encode membrane associated or secreted proteins are characterized by a striking degree of variability (Murphy et al. 2003; Pignatelli et al. 2001; Dolan et al. 2004). The data in this thesis (Fig. 6) show that the increased NKG2H expression by PBMCs exposed to HCMV infected fibroblasts was dependent not only on the type of the virus (HCMV, but not HSV or Vaccinia), but also on the strain of HCMV employed. Of the four HCMV strains that were studied here, Merlin, Toledo, TB/40 and AD169 only HCMV strains Merlin and TB/40 were able to drive the increase of NKG2H expression. From the low-passage clinical

strains, the minimally passaged strain Merlin is considered to best represent the wild-type HCMV genome, containing no obvious mutations other than a single nucleotide substitution that truncates the UL128 gene (Dolan et al. 2004). The highly-passaged, attenuated strain AD169 is known to have lost virulence and experienced substantial alteration in its genome during passage in cell culture (Elek et al. 1974; Quinnan et al. 1984) including the loss of a region of DNA containing at least 19 ORFs (denoted UL133 to UL151) that are normally found in the low-passage clinical isolates including, Toledo, TB/40 and Merlin (Cha et al. 1996). Thus there is a possibility that some of these genes, at least in the case of TB/40 and Merlin are important for the increased NKG2H expression. It has been recently demonstrated that HCMV infection of different monocyte-derived cell types triggered different NK cell response (Romo et al. 2011[(2)]). Additionally, depending on the degree of adaptation in fibroblasts, HCMV strains display different levels of virulence, tissue tropism, and pathogenicity. All four of the HCMV strains used in these studies have been sequenced and these analyses have revealed divergences in genome-size, inversion, orientation and coding potential, even within conserved genes, including nucleotide polymorphism, DNA strand composition asymmetry, and evolutionary rate variation in conserved genes (Murphy et al. 2008; Cunningham et al. 2010). Thus it will not be trivial to elucidate the key differences between Merlin/TB40 and Toledo/AD169 that underlay the differential ability of these strains to drive increased NKG2H expression, particularly since the differences in the ability of these viruses to trigger increased expression of the NKG2H receptor after exposure to virus-infected hTERT fibroblasts, could reflect some difference in the direct interaction of a viral component with the NK cell or, some difference in the replication of these viruses in fibroblasts that indirectly modulates this phenotype. For example, diversity in the requirements for the function of some core DNA replication proteins involved in HCMV DNA replication during lytic infection have been described between different fibroblast cell lines (Goldmacher et al. 1999; Skaletskaya et al. 2001; Xu et al. 2004). Finally, we observed no change in the proportion of $NKG2H^+$ cells in PBMCs co-cultured with HSV and Vaccinia virus infected fibroblast strongly suggesting that the expansion of CD94/NKG2H expressing cells, at least to some extent, is HCMV- specific.

Interestingly, when the expression of NKG2H mRNA by the virus stimulated PBMCs was analysed no correlation between transcription levels and surface expression of NKG2H molecule was seen. This suggests that transcriptional regulation does not account for the enhanced expression of NKG2H molecule and indicates as more likely a post transcriptional and/or posttranslational regulation of NKG2H protein expression at the cell surface. Consistent with this idea, recently, data have been published demonstrating the existence of intra-cytoplasmic complexes of NKG2E with CD94 and DAP12 (Orbelyan et al. 2014). NKG2H is a splice variant of NKG2E in which intron VI has not been removed and does not contain exon VII. Thus the existence of NKG2H protein and/or CD94-NKG2H-DAP12 complexes constitutively stored in intra-cytoplasmic pools that can be expressed after specific receptor–ligand interaction in the absence of new gene expression might be possible mechanism of regulation of surface expression of NKG2H. Experiments addressing the mechanisms involved in the regulation of constitutive and inducible NKG2H expression need to be done.

Withdrawal of PBMCs from contact with the infected fibroblast led to a rapid loss of NKG2H expression supporting the idea that, under the influence of HCMV infection, CD94/NKG2H is only transiently induced at the cell surface. This observation is consistent with the hypothesis that acquisition of the NKG2H activating receptor may play a role in the regulation of T cell and NK cell responses during the acute phase of virus infection so that on apparent resolution of HCMV infection, NKG2H expression is no longer necessary and the receptor disappears from the cell surface. However, a recent report suggests that HCMV independently affects the NK and T cell compartments (Bengner et al. 2013), thus the possibility that NKG2H molecule may play different roles in individual cell subsets should not be excluded.

In order to assess the functional capacity of PBMCs after co-culture with infected fibroblasts CD107a degranulation assays to measure NK (Alter et al. 2004) and T cell (Kannan et al. 1996; Bossi et al. 1999; Betts et al. 2003) cytotoxicity were performed. The increased percentage of $CD107a^+$ cells in PBMCs stimulated with mock infected fibroblasts was due to allogeneic responses made by the donor PBMCs towards the HLA-mismatched fibroblasts and includes responses of both allo-reactive T cells (Lindahl et al. 1977; Kalil et al. 1983; Nieda et al. 1985; Suchin et al. 2001) and NK cells (Ciccone et al. 1992[(1)(2)]; Colonna et al. 1992; Colonna et al. 1993). It has been previously proposed that the presence of cross-reactive anti-viral T cells may contribute to a less controllable and easily magnified immunological response to allogeneic stimulations and/or viral reactivation (Gamadia et al. 2004; Elkington et al. 2005; Rist et al. 2009; Morice et al. 2010). According to our data, neither $CD3^-$ $CD56^+$ NK cells nor $CD3^+CD56^-$ T cells or $CD3^+CD56^+$ cells previously exposed to HCMV infected fibroblasts were primed for enhanced reactivity towards the HLA-allo-antigens on the mock-infected cells or the HCMV infected fibroblasts on a second exposure to viral antigens. In contrast, T cells previously exposed to mock infected fibroblasts showed enhanced responses to those same fibroblasts on secondary challenge, but responded very poorly to HCMV-infected fibroblasts. NK cells primed by culture on mock infected fibroblasts showed a marked increase in their degranulation to HCMV infected cells (and a smaller increase towards mock infected fibroblasts). According to our data, the exposure of PBMCs to HCMV infected fibroblasts triggered an increase in the proportion of $NKG2H^+$ cells for both T and NK cells, thus one interpretation of these data is that the increased expression of NKG2H has different effects on T and NK cells; inhibition of T cell activation, but enhancement of NK cell stimulation. These experiments are however very complex, since HCMV infection will downregulate MHC class I expression – probably affecting the priming of alloreactive T cells and HCMV infection will also lead to the downregulation of multiple ligands for activating receptors of NK cells again affecting the activation of these cells. Indeed HCMV infection protects allogeneic fibroblasts from NK cell recognition (Valés-Gómez et al. 2003; Wilkinson et al. 2008). Moreover at present no firm conclusions can be made about the role of NKG2H in the phenomena observed. Thus, this experiment needs to be repeated and improved. A first step would be to include $F(ab)_2$ fragments of blocking CD94 mAbs and ideally an NKG2H mAb. An additional complication is that modification of the currently available NKG2H-specific mAb, by labelling with Alexa 488, PE or biotin, leads to loss of reactivity (data not shown), presumably reflecting the presence of a lysine residue in the antigen-combining site of this mAb that is targeted by the labelling

chemistry. The absence of a directly labelled NKG2H-reactive mAb renders impossible the study of NKG2H-expressing lymphocytes in experiments where blocking antibodies are used.

To further explore the functional consequences of NKG2H engagement for cells that endogenously express this receptor, we compared the effects of stimulation of purified PBMCs with anti-CD3, anti-NKG2H and anti-CD3/NKG2H antibody mixes immobilized on plastic. The experimental set-up used: anti-NKG2H antibody stimulation in the presence and absence of suboptimal doses of stimulatory anti-CD3 antibody allowed the detection of both enhancement and reduction of the stimulation triggered by CD3 cross-linking. Under these conditions NKG2H ligation resulted in a profound inhibitory effect on T cell activation in these cultures. This was confirmed by the evaluation of expression of early activation-associated marker CD69, which appeared to be significantly lower on stimulation of PBMCs after NKG2H receptor ligation. Moreover, this inhibitory effect was specific for NKG2H receptor signalling as stimulation of PBMCs with either anti CD3/NKG2A or anti-CD3/NKG2C antibody mixes resulted in comparable levels of expression of CD69 as the PBMCs receiving control anti-CD3 stimulation only. These data, together with the flow cytometry observation of preferential expression of the NKG2H receptor on $CD3^+$ cells, implies a possible negative regulation of T cell activation mediated by NKG2H receptor signalling. This observation is particularly striking since NKG2H is expressed by only a small fraction of all the T cells in the culture that could respond to CD3 cross-linking.

Experiments using culture supernatants collected after anti-CD3/NKG2H stimulation of PBMCs revealed that the suppressive activity could not be accounted by secretion of soluble factor(s) from NKG2H stimulated cells. This requirement for cell contact for NKG2H to mediate inhibition suggests that these cells act directly on other T cells to prevent activation. Simultaneous CD3/NKG2H receptor ligation suppressed the proliferation of the PBMCs in the culture and the NKG2H-dependent suppression could be shown to be due to the induction of T cell apoptosis. The simplest interpretation of these observations is that the subsets of T cells that express NKG2H exert a negative effect on T cell activation, leading to the subsequent induction of cell apoptosis. It is still not clear whether this effect is mediated by upregulation of NKG2H expression after TCR-stimulated activation followed by NKG2H ligation and cell intrinsic inhibition or whether the lymphocytes that express NKG2H prior to stimulation become able to inhibit the activation of other T cells *in trans*. Either scenario would explain the failure of multiple attempts to expand populations of T cells enriched for NKG2H expression using either FACS or MACS based cell sorting, with or without single cell cloning followed by growth of the purified $NKG2H^+$ T cells *in vitro* in the presence of irradiated autologous PBMCs, supplemented with exogenous IL-2 or combinations of IL-2 and the mitogen PHA (data not shown). Alternatively it cannot be excluded that the proliferative capacity of $NKG2H^+$ T cells is limited and/or that specific co-/stimuli and/or cytokines are necessary to enable these cells to divide. Such regulation has not been observed for other activating NKR such NKG2C (Guma et al. 2005; Meresse et al. 2006) but however, it would be reminiscent of some features of the CTLA-4/B7 regulatory loop (Perkins et al. 1996).

It is interesting to note that the CD94/NKG2H receptor was described as binding the adaptor molecule DAP12. Although, DAP12 was initially described in NK cells in humans expression of the adaptor molecule by T cells was initially somewhat controversial. However, $CD4^{+}CD28^{-}$ T cells that express both DAP12 and activating KIR have been described in patients suffering from chronic inflammatory diseases (Snyder et al. 2003; van Bergen et al. 2004). Furthermore, in spite of DAP12 being first thought of as an ITAM-containing adaptor molecule for activating receptors it has subsequently been shown that the function of DAP12 is more complex than originally thought. DAP12 associated receptors can downregulate TLR-dependent responses in macrophages as well as CD16-dependent responses in NK cells (Hamerman et al. 2005; Hamerman et al 2006). Similarly, DAP12 down-modulates the cytokine production by plasmacytoid dendritic cell (pDC) in vivo during murine cytomegalovirus infection (Sjolin et al. 2006) and DAP12-deficient B cells are hyper-responsive after stimulation with anti-IgM or CpG, suggesting that DAP12-coupled receptors negatively regulate B cell-mediated adaptive immune responses (Nakano-Yokomizo et al. 2011). It has been proposed that the cellular response signalled by DAP12 (activation or inhibition) varies in function of the avidity of the interaction between the DAP12-associated receptor and its ligand (Turnbull et al. 2007). It is also worth noting that our findings differ from those reported by Bellón et al. 1999 because in their work aggregation of the putative CD94/NKG2H heterodimer expressed on a T cell clone triggered cytotoxicity and IFN-γ production in a TCR-independent manner. This discrepancy might simply reflect that in their experiments mAb mediated cross-linking of CD94 receptor was used and the presence of an activating NKG2C molecule on the clone was never excluded, whereas in our experiments stimulation of NKG2H by a specific monoclonal antibody was applied. It is also possible that the responses of a single T cell clone selected for long-term growth in *in vitro* culture may not be representative of the full spectrum of responses of freshly isolated peripheral blood T cells in short-term culture. In summary then, it is possible that NKG2H can exert either stimulatory or inhibitory effects on T cells and perhaps the level of receptor cross-linking is extremely important for generating negative signalling. Further experiments will be required to determine the important factors influencing NKG2H-mediated interactions with other cells, including the level and type of NKG2H ligand expression, the expression of cofactors, and the ratio of interacting cells.

Given that the best characterized CD94/NKG2 heterodimers expressed by NK cells recognize HLA-E molecules loaded with nonapeptides derived from HLA-class I signal sequences (Borrego et al. 1998; Braud et al. 1998; Lee et al. 1998; Llano et al. 1998) one obvious candidate ligand for the NKG2H receptor would be HLA-E. However Bellón et al. 1999 have reported that the TAP-deficient mouse cell line RMA-S when transfected with HLA-E cDNA (Borrego et al. 1998) and cultured in the presence of peptides that stabilize HLA-E on the surface was not recognized by the T cell clone expressing NKG2H. Similarly, in the experiments described in this thesis, addition of an anti-HLA class I mAb (HP-1F7), which detects all classical and nonclassical HLA class I molecules including HLA-E (Pérez-Villar et al. 1997; Lee et al. 1998; Takao et al. 2010), failed to recover the activation of the PBMCs in the cultures stimulated with anti-CD3/NKG2H antibodies. Finally, although it was suggested that the splice variant NKG2H, would behave identically to NKG2E and probably bind

to HLA-E with similar affinity (Kaiser et al. 2005) so far, there are no data that demonstrate an interaction between HLA-E and NKG2H. In aggregate therefore, the available data suggest that it is possible that NKG2H recognizes and binds molecules that are not related, at least to conventional HLA-E-leader peptide loaded complexes. However, to date all attempts to produce soluble NKG2H molecules, which would greatly facilitate the search for possible ligands for this receptor, have been unsuccessful. Difficulties in expression and refolding of wild type intact ectodomain of NKG2E molecule have also been documented (Kaiser et al. 2005) thus new strategies to achieve high level expression of this gene *in vitro* are required in order to generate the reagents needed to try and define the ligand(s) for this receptor.

HLA-E tetramer guided detection of *Mycobacterium tuberculosis* – specific $CD8^+$ T cells in bladder cancer patients receiving BCG therapy.

Although immunotherapy by intravesical instillation of live bacilli Calmette Guerin (BCG) has proved to be an effective treatment for non-muscle invasive bladder cancer (Morales et al. 1976; Sylvester et al. 2002; Herr et al. 2008) the mechanism of action of intravesical BCG therapy is still not well understood, although it is clear that the induction of a strong innate immune response as well as T cell infiltration into the bladder are important. As discussed previously, one aspect of this therapy that has proved particularly controversial is whether immunity to tuberculosis antigens is of benefit to the patient; why should an immune response to a bacterium lead to elimination of a tumour cell? It has been speculated that the pathogen invades the cancer cells and once inside leads to their destruction, perhaps by stimulating some form of antitumor immunity (Redelman-Sidi et al. 2014). An alternative view is that exposure to BCG stimulates a non-specific inflammatory response that somehow, perhaps by altering the balance of cytokine production, indirectly potentiates the generation of effective anti-tumour immunity (Redelman-Sidi et al. 2014). If the hypothesis ¨immune responses to BCG benefit the patient¨ is valid, then rounds of treatment with BCG instillation are likely to be associated with an enhanced Mtb-specific adaptive immune response. The importance of non-classically restricted T cells in the host response to infection with *M.Tuberculosis* has already been addressed (Heinzel et al. 2002), however the possible role of *M.Tuberculosis* – HLA-E - restricted T cells induced in bladder cancer patients after BCG instillation therapy has not been addressed. Using Mtb-p62 and *Mtb*-p68 HLA-E tetramers we screened nine bladder cancer patients receiving BCG therapy for possible Mtb-peptide reactive T cells. Our data demonstrate low frequencies of $tetramer^+$ cells in the peripheral blood from these patients although in some of them, detectable levels could be observed during the period of rest for two months. The frequencies of tetramer reactive cells detected are comparable to other systems where tetramers have been used to visualize antigen-specific T cells. However, it might be expected that, the numbers of tetramer stained T cells could vary over time, perhaps decreasing during the two months rest phase and increasing after the rounds of instillation of BCG and such a relationship was not observed. Obviously, for ethical reasons, in these experiments the staining was done using peripheral blood lymphocytes and if activated HLA-E restricted T cells infiltrate into the bladder after intravesical instillation of live BCG then it might not be surprising that only a very low percentage of these cells could be

found circulating in the bloodstream and that the kinetics of their appearance does not coincide with the application of the therapy. Another factor to take into account is that even though the HLA-E restricted epitopes used in this study were commonly recognized by donors exposed to Mtb (Joosten et al. 2010), it could be that the pattern of epitopes recognized after intravesical BCG instillation is different or that intravesical instillation of BCG does not efficiently prime systemic T cell responses. Finally, the BCG vaccination status of the bladder cancer patients is currently not known, thus it is possible that the tetramer$^+$ cells detected in the blood of some of the patients before receiving their first instillation of BCG are memory T cells generated due to exposure to Bacillus Calmette Guerin (BCG) vaccine or *M. tuberculosis* infection.

Although HLA-E is considered to be a highly conserved molecule, the recognition and binding of certain Mtb derived peptides was shown to be diverse and the capacity of these peptides to be presented and trigger a response by CD8$^+$ T cells was donor related (Joosten et al. 2010). In our study, we used only 2 from 69 predicted and characterized Mtb-derived peptides to generate recombinant HLA-E-peptide complexes *in vitro*. A larger study, where the same technology is applied, but including additional HLA-E binding Mtb-derived peptides is required. Extending the analysis to include more bladder cancer patients, with known PPD status, would also increase the possibilities to discover and characterize the significance of Mtb-peptide-HLA-E specific T cells after BCG instillation as a biomarker for treatment outcome in bladder cancer patients receiving BCG therapy. In this context, it is worth pointing out that the prognostic significance of the expression of HLA-E by bladder cancer cells is not very well characterized. In contrast to HLA-G, less information is available regarding HLA-E expression by malignant cells (Wischhusen et al. 2007). In surgically removed malignant lesions, HLA-E expression is increased in glioblastoma, carcinomas of colon and ovary, lymphoma and melanoma (Lee et al. 2003; Chang et al. 2005; Wischhusen et al. 2007; Campoli et al. 2008). As immune recognition of a cancer cell depends not only on the level of tumour MHC class I expression, but also on the molecular mechanisms which cause alterations in the MHC class I expression (Aptsiauri et al. 2013), then even if HLA-E is expressed by the bladder cancer cells the ability of these molecule to bind and express at the cell surface Mtb peptides after BCG treatment could be altered.

Lastly, the failure to detect enhanced Mtb-tetramer reactivity could simply reflect that, although HLA-E restricted CD8$^+$ T cell responses are found in Mtb infection, these responses may not be generated efficiently in these bladder cancer patients. In this regard, this study might be indicating that the concept of an important role for BCG specific antigen MHC-restricted response as a key mechanism of BCG mediated anti-bladder cancer immunity is erroneous. An important caveat to this conclusion is the lack of a really good positive control for the Mtb-tetramer staining. However, data from other members of the group suggest that the interaction of BCG with PBMC leads to NK cell activation and enhanced tumour cell killing by these activated NK cells, and these observations are consistent with data from murine models demonstrating a key role for NK cells in immune responses to bladder cancer (Brandau et al. 2001).

It is also interesting to note that expansions of $CD8^+$ T cells reactive with the HLA-E/B7 tetramer were observed in some patients during the course of treatment. The expectation is that this tetramer will bind with highest affinity to CD94/NKG2A heterodimers (Valés-Gómez et al. 1999). Thus the staining with this tetramer probably corresponds to $CD8^+$ T cells that express inhibitory CD94/NKG2A receptors. When the changes in cell surface expression of the CD94 molecule on T cells in the blood from these patients was analysed and the attempt was made to relate these data to the HLA-E/B*07 $tetramer^+$ staining of $CD8^+$ T cells. Significant variability between the different donors both in the percentage of cells expressing CD94 as well as variation in the populations of T cells expressing different amounts of the CD94 molecule on the cell surface was found. Different levels of expression of CD94 on T and NK cell clones has previously been shown to correspond to expression of inhibitory ($CD94^{high}$ $\alpha\beta^+$ T cells are all $NKG2A^+$) and activating ($CD94^{dim}$ T cells either express NKG2C and/or NKG2E or NKG2H or lack transcripts altogether) CD94/NKG2 heterodimers (Perez-Villar et al. 1996; Jabri et al. 2003). However analyses of the bladder cancer patients revealed that some of the patients maintained other subpopulations e.g. $CD94^{high++}$, while in others it was not possible clearly to separate $CD94^{high}$ from $CD94^{dim}$ subpopulation. Thus, although some degree of correlation between the expansions of $CD8^+$ T cells reactive with the HLA-E/B*07 - tetramer and the change of the cell surface expression of $CD94^{high+}CD3^+CD56^-CD4^-$ cells could be seen in some of the patients, the complexity of the data analyses and the small sample size make it difficult to make any strong conclusion just now.

The significance of induced expression of immunoregulatory molecules such as CD94/NKG2A on $CD8^+$ T cells is not fully understood, but it is generally thought that these receptors restrain $CD8^+$ responses. While negative regulation of CTL responses may be beneficial in infections by preventing T-cell exhaustion in chronic infections and limiting immunopathology it might also be the case that limitation of the immune response in cancer does not lead to a good outcome; one possible analogy might be the role of inhibitory molecules such as CTLA-4 and PD-1 in modulating immune responses in melanoma and other cancers (Kyi & Postow, 2014). Another example is cervical cancer-infiltrating $CD8^+$ T lymphocytes, which express increased CD94/NKG2A levels compared with peripheral blood $CD8^+$ T cells and/or normal cervix-infiltrating $CD8^+$ T lymphocytes. Such increased expression of inhibitory NKRs could be triggered via IL-15 or TGF-β mediated mechanisms and probably abrogates the antitumor cytotoxicity of the tumour infiltrating lymphocytes (Sheu et al. 2005). An increased frequency of CD94 and NKG2A expression by tumour infiltrating lymphocytes compared to peripheral blood T cells in human endometrial carcinoma may also shape the cytotoxic response of these $CD8^+$ T cells, which may again suggest a possible mechanism of tumour escape from host immunity (Chang et al. 2005). In the context of the group of bladder cancer patients studied, it is not currently possible to know whether this putative restraint of immune responses has a net positive or negative effect, but it is particularly striking that $CD8^+$ T cells of only a subset of patients seem to express CD94/NKG2A since, while BCG therapy leads to tumour clearance and patient survival in around 70% of patients the tumours of around 30% of patients do not respond to this therapy. Thus it will be interesting in future analyses to include anti-NKG2A/NKG2C or

NKG2H antibody together with the other T cell surface markers, which could probably help to further classify patients and especially compare the clinical outcome of those patients that have experienced CD94/NKG2A upregulation on $CD8^+$ T cells with those who have not.

Conclusions
Conclusiones

The studies on the NKG2H receptor have shown:

1. In the peripheral blood of healthy individuals, the CD94/NKG2H receptor is expressed on low numbers of circulating lymphocytes, principally on $CD3^+CD56^+$ and $CD3^+CD8^+$ cells.
2. The frequency of $NKG2H^+$ cells increases when PBMCs are co-cultured with HCMV infected fibroblasts. No significant changes in the percentages of NKG2A or NKG2C expressing T cells, NK cells, and $CD3^+CD56^+$ cells were observed after culture of PBMCs with the virally infected fibroblasts.
3. The elevated proportion of NKG2H expressing cells produced by contact with HCMV infected fibroblasts is not associated with increased levels of mRNA for NKG2H. Therefore, the surface expression of NKG2H is most likely regulated post-transcriptionally and/or post-translationally.
4. The increase in the frequency of $NKG2H^+$ cells depends on the type and genetic variant of the virus.
5. Direct contact between the lymphocytes and the HCMV infected fibroblasts is required for optimal induction of $NKG2H^+$ cells. The increased fraction of NKG2H expressing cells is not stable over time since NKG2H expression is lost after separation of PBMCs from infected fibroblasts.
6. Stimulation of cells via NKG2H, but not through the NKG2A or NKG2C receptors, induces apoptosis and notably reduces the activation and proliferation of the other cells in the culture.
7. The NKG2H dependent reduction of cell culture activation does not depend on soluble factors or recognition of MHC class I molecules.

The studies using peptide/HLA-E complexes to analyse the PBMCs of bladder cancer patients have shown:

8. The frequencies of HLA-E/p62 and HLA-E/p68 $tetramer^+$ $CD8^+$ T cells in the peripheral blood from 9 bladder cancer patients receiving BCG immunotherapy are low.
9. An expansion of $CD8^+$ T cells reactive with HLA-E/B*07-tetramer could be observed in some bladder cancer patients during the course of treatment with live BCG. Once the clinical outcome of these patients is resolved it will be possible to evaluate whether there is any relationship between these changes and response to therapy.

Los estudios sobre el receptor NKG2H han mostrado:

1. En sangre periférica de individuos sanos el receptor CD94/NKG2H se expresa en un porcentaje bajo de células $CD3^+CD56^+$ y $CD3^+CD8^+$.

2. La frecuencia de células NKG2H positivas aumenta tras el co-cultivo de PBMCs con fibroblastos infectados con HCMV. Sin embargo, no se observan cambios significativos en los porcentajes de células T, NK ni $CD3^+CD56^+$ que expresan NKG2A o NKG2C tras el mismo co-cultivo usando sangre periférica de donantes seronegativos para HCMV.

3. La elevada proporción de células expresando NKG2H que se observa tras el contacto con fibroblastos infectados con HCMV no se asocia con niveles elevados de mRNA para NKG2H. Por lo tanto, la expresión en superficie de NKG2H probablemente esté regulada por modificaciones post-transcripcionales y/o post-traduccionales.

4. El incremento en la frecuencia de células NKG2H positivas depende del tipo y la cepa del virus.

5. Se requiere contacto directo entre los linfocitos y los fibroblastos infectados por HCMV para la inducción óptima de células NKG2H positivas. El incremento en la fracción de células que expresan NKG2H no es estable con el tiempo, y la expresión se pierde tras la separación de los linfocitos y los fibroblastos infectados.

6. La estimulación de células a través de NKG2H pero no NKG2A o NKG2C induce apoptosis y reduce notablemente la activación y la proliferación de otras células en el cultivo.

7. La reducción de la activación en el cultivo celular dependiente de NKG2H no requiere factores solubles, ni el reconocimiento de moléculas MHC de clase I.

Los estudios utilizando complejos péptido/HLA- para analizar PBMCs de pacientes de cáncer de vejiga han mostrado:

8. La frecuencia de células T $CD8^+$, positivas para los tetrámeros HLA-E/p62 y HLA-E/p68, es baja en sangre periférica de nueve pacientes de cáncer de vejiga recibiendo inmunoterapia con BCG.

9. Se observa una expansión de células T $CD8^+$ reactivas con el tetrámero HLA-E/B*07 en algunos de los pacientes de cáncer de vejiga a lo largo del tratamiento con BCG. Una vez que se conozca la respuesta a la terapia de cada paciente será posible evaluar si existe alguna relación con los cambios observados.

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Acknowledgment/ Признание и Благодарности

Well…

Here I am, scratching the final words, and the most difficult one look to be ….

Mar, Hugh !!! I OWE YoU A SPEcIAL ThankS; required a courage to believe in me and shake my hand when I felt that this road at one point probably was finished; Time was not essential to see your optimism, great support and encouragement. Thank you for every ¨sure¨, ¨not a problem¨, ¨any time¨. For ideas, always framed in orientation and rigor; for the great knowledge and amazingly simple, comprehensive and engaging teaching that you practice every day. No solo habéis confiado en mi, además me rodeastéis con un ambiente y una ilusión en el trabajo en el que me he sentido muy muy bien y del que siempre os estaré agradecida. De corazón.

Of course, there aRe all the people that I had chance to meet during these years.

First I want to say THaNKs *a mis primeras compañer@s, el gran equipo del labo 414-417: a Glo por su sonrisa siempre disponible y ese carisma tan alegre, durante tooooodos y cualquier día. Gema, por toda esa paciencia que has tenido conmigo (y además en inglés), recuerdo al principio cuando me enseñabas como funcionaba la vida en el labo, gracias, además siempre has tenido momentos para mi (y para todos). Miriam, por las opiniones sobre mis westerns I su kikoteo. Eva por la cena científica mas extraña que tuve, que jamás olvidaré ☺ , la que más sufrió con el cambio de Spanish a Enlglish, thanks ☺, y con esa velocidad turbolente (casi como yo ☺). Sheila, por ver siempre el lado positivo y tener su forma de mostrarme que ¨no pasa nada mujer¨, eres inolvidable. A Lola, la mas aventurera y ¨crazy¨ de todas, siempre con algo que contar. Chicas gracias por la paciencia y sobre todo por aceptarme, encima como yo soy, la menos sociable, pero cada vez mas comunicable porque y gracias a vosotras, en vuestro alrededor es imposible no hacerlo, imposible no abrirse (con lo que me costabaaa!!!!). Uf, la amistad que tenéis y siempre habéis estado dispuestos a entregar y ofrecer ha sido lo mejor y más grande. Gracias. Y* gracias al resto de personas que han pasado por el labo y han dejado su huella, Rachele, por traer la sonrisa cada vez que entraba en 417 para saludar (su acento español me encantaba), Bea, Jose Ramon por su encanto de persona y grandeza. A Juan Fran, a Alfonso por su cariño y por ser una de las personas más luminosas que he conocido, a Dani no solo porque comparte mi nombre (¨Dani¨ mi firma por todas las hojas al apuntarse para los servicios del departamento) y también por la delicadeza de su cariño y ayudarme llevando mi trabajo adelante. Daria, you get to know me at the point of craziness, writing the thesis. Thank you for your short pleasant conversation whenever was necessary for me to disconnect from the thesis world. Ruth con la que siempre encontrábamos algo de charlar.

I also want to thank the Cytometry service for their kindness and for making my days, and there were lots of hours, pleasant leafing between each tube. I would like to extend my gratitude to all the people that I met in the department and centre.

Благодарам до моите родители, мама и тато. Секогаш километри далеку а блиску до мене. До сестра ми, која секогаш ме држи за рака. До

Вики, мојата втора " сестра" поради поминати часови и часови разговор полни со подршка и разбирање во моменти на криза и паника и без која не би била сега тука. До Бибе, мојата другарка од факултет која од Тарагона секогаш успева да ме насмее. До сите мои пријатели кои во овие години константно ми недостигаат. И благодарам на Виктор, што го запознав, бакнав, сакам со цело срце и со кого иднината е се.

Благодарам, Thank you, Gracias ¡!!